SPECIFIC INFECTIOUS CYSTITIS AND PYELONEPHRITIS OF COWS.

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Plates 26 to 28.

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Various bovine infections characterized by a pyelonephritis have been recognized in Europe.

Guillebeau[1] described a condition of such nature in 1888, and several have since reported diseases affecting the pelvis of the kidney. We would infer from the literature that renal diseases among cattle are widespread in Continental Europe. A number of etiological agents have been recognized. Höflich[2] and Enderlen[3] both cultivated from the affected kidneys of a few cases a Gram-positive diphtheroid which Enderlen names *B. pyelonephritis boum.* Ernst,[4] who studied the kidney lesions from a series of cases, encountered a similar organism in a certain number; while from others he obtained *B. pyogenes* and *B. coli.* Owing to the difficulty encountered in reproducing the disease in cows with organisms similar to those isolated by Höflich and Enderlen, considerable confusion seems to have arisen. Ernst, from certain experimental observations, even questions the etiological relationship of the diphtheroid to the processes encountered. Joest,[5] however, considers pyelonephritis a specific infectious disease of cattle and calls attention to the fact that there is no disease having a similar etiology and pathology.

We are not aware that diseases of this nature have been reported in this country.

The Appearance of Pyelonephritis in New Jersey.

In October, 1924, our attention was called to a cow affected with nephritis. At the owner's request the cow was autopsied. Lesions of

[1] Guillebeau, *Schweiz. Arch. Tierheilk.*, 1890, xxxii, 224.

[2] Höflich, D., *Monatsh. prakt. Tierheilk.*, 1891, ii, 337.

[3] Enderlen, D., *Z. Tiermed.*, 1890–91, xvii, 325.

[4] Ernst, W., *Centr. Bakt., 1. Abt., Orig.*, 1905, xxxix, 549.

[5] Joest, E., Spezielle pathologische Anatomie der Haustiere, Berlin, 1924, iii.

pyelonephritis were noted, and a diphtheroid was isolated from the kidney. A more detailed report of the pathological findings will be given later. It was possible to obtain a relatively complete history of importations into this herd during the past few years, and in addition, a record had been kept of the deaths and the autopsy findings. In November, 1922, a number of cows were purchased in the Middle West. The first loss from nephritis occurred in April, 1923. Another cow died in May, 1923. During 1924 two cows died of the disease, one in July and the other in August. We autopsied a severe case (No. 1132) on October 27, 1924. Since then other cases have been recognized.

A larger herd in which the practice is to introduce a large number of cows each year has been under observation by various members of the staff for a considerable period. Infections of the kidney were known to exist; in two instances[6] in which bacteriological examinations had been made *Bacillus pyogenes* was obtained. Inasmuch as there had been several importations during 1922 from the same district as that referred to in the smaller herd, search was instituted for such infections in the larger herd.[7] On November 9, 1924, a cow (No. 1138) was found dead. Bacteriological examination of the kidneys revealed an organism similar to that obtained from the case mentioned. Between November, 1924, and May, 1925, twenty such infections have been recognized. Since certain cases illustrate points to be considered further on the protocols of a number will be given.

No. 1132.—A Holstein cow, one of the smaller herd referred to, had been purchased in the Middle West in 1922. It had given birth to a calf in August, 1923. On Oct. 20, 1924, the milk flow suddenly lessened. On Oct. 27, the cow was examined. Unthriftiness was noticed. The temperature was 102°. The appetite was good and the feces normal. Weakness was pronounced. The pulse and respiratory rate were normal. A little turbid urine was obtained. The cow was slaughtered and autopsied at once.

The lesions were confined to the urinary apparatus. Both kidneys were enlarged. The cortex of the right kidney was clay color. The consistency was firm and tough. Scattered over the surface were many irregular, slightly raised, gray patches. They varied in size from barely visible points to patches which occupied

[6] We are indebted to Dr. Theobald Smith for these data.

[7] Since the data have been gathered together the disease has been encountered in a third herd.

practically a whole lobe. The capsule was adherent over such areas. On section the processes frequently extended through the cortex and into the medulla. The medulla was bright red except in the portions where the gray patches had extended from the cortex. The pelvic space was dilated and filled with a greenish yellow purulent exudate. The left kidney presented similar lesions. Both ureters were increased in diameter. The left measured 2 cm. in diameter. The walls were thickened and the mucosa congested. An exudate similar to that observed in the kidney pelvis was present in the ureters. The bladder was contracted. It contained 200 cc. of turbid urine. The walls were extremely thick. The mucous membrane was partially covered with an adherent, rough, grayish white exudate. The urethra presented no gross lesions. The uterus was apparently normal. A specimen of urine obtained from the bladder contained considerable yellowish white stringy material. Examination showed the following: Reaction, alkaline; specific gravity, 1.016; albumen, present (Esbach's, 0.4 per cent); sugar, not present. Leucocytes and bacilli in enormous numbers were present in the sediment.

Inoculations into various media were made from the heart blood, spleen, and kidneys. The tubes inoculated with heart blood remained sterile. Bits of both kidneys implanted in the condensation water of blood and plain agar slants developed characteristic diphtheroids both in pure culture and mixed with streptococci. In all instances the diphtheroids were present in large numbers. One tube inoculated with a fragment of spleen developed the same organism in pure culture. Films and fresh preparations from the urine and the exudate from the pelvis of the kidney revealed, on microscopic examination, the diphtheroids in large masses.

Fixed and stained sections from the spleen and uterus revealed no abnormalities on microscopic examination. Many of the liver cells were granular; some were infiltrated with fat. The sections from the kidneys presented severe lesions. The cortex had been invaded by densely packed masses of round cells. In places considerable of the cortical structure had been practically obliterated by the infiltrative process. Leucocytes in dense masses were frequently observed throughout the cortex. Deep within the cortex connective tissue hyperplasia had occurred. Leucocytes in large numbers had invaded the connective tissue. The adjacent tubules were compressed and their epithelium degenerated. In certain portions of the medulla considerable interstitial hyperplasia existed. The collecting tubules were compressed and frequently filled with epithelial casts. In other portions the process was more acute. The blood vessels were engorged and the tubular epithelium degenerated. Often the tubules were plugged with polymorphonuclear leucocytes and fibrin. The interstitial tissue was edematous.

Much of the mucosa of the ureters was necrotic or degenerated. The free surface frequently consisted of a mass of granular exudate containing leucocytes, red cells, and necrotic epithelium. In portions where the process was not as advanced many of the epithelial cells were degenerated and leucocytes in large numbers had invaded the mucous layer. The submucous connective tissue was

edematous and the capillaries engorged with red cells. The edema extended into the muscular layers.

No. 1138.—Purchased in the Middle West Aug. 7, 1922. Sept. 29. Calved normally. Jan. 29, 1924. Bred. Aborted Sept. 23. Died Nov. 9. On Nov. 10, both kidneys, the bladder, and the uterus were submitted for examination. There was considerable decomposition.

The right kidney was enlarged. It measured 25 × 15 × 5 cm. The color was yellowish gray. The consistence varied; in places it was firm, in others softened. On the whole both the cortex and medulla appeared narrower than normal. The line of demarcation between the cortex and medulla was indistinct. The more superficial portions of the medulla were bright red. Deeper the medullary structures appeared necrotic. The pelvis was greatly dilated and partially filled with necrotic material, and blood-stained urine.

The left kidney was also enlarged. It measured 23 × 13 × 6 cm. The color was yellowish red. The consistency was tough. On section the color varied from yellowish pink in the cortex to deep red in the medulla. The papillæ were necrotic. The pelvis was dilated and filled with blood-stained urine and foul smelling exudate.

Only small portions of the ureters were obtained. They were greatly dilated. The walls were appreciably thickened. Granular tufts were adherent to the mucous membrane. The tubes were partially occluded with yellow exudate.

The bladder was contracted. It contained 300 cc. of red-stained urine. The mucous surface was reddened and swollen; patches of grayish white exudate adhered to the mucosa. The urine obtained from the bladder was turbid and of a deep red color. The specific gravity was 1.020. The reactions for albumen were strong (Esbach's, 1.5 per cent). The urinary sediment contained red cells, leucocytes, cocci, and diphtheroids in large numbers.

There was marked decomposition of the uterus. The walls were moderately thickened. The mucosa was necrotic and covered with a foul smelling, greenish white exudate.

Cultures taken from the kidney cortex showed only the presence of anaerobic putrefactive types. Those from the kidney pelvis and urine developed cocci in small numbers and diphtheroids in enormous numbers. The uterus contained an enormous number of bacteria. *B. pyogenes* predominated but diphtheroids similar to those found in the urine and kidneys were also found in limited numbers.

Inasmuch as the material was not fixed in Zenker's until decomposition had taken place the sections were not particularly illuminating. The kidney cortex contained well defined wedge-shaped areas infiltrated with round cells. The glomerular epithelium was degenerated and congested. The tubular epithelium in certain portions appeared as granular, pink-staining cells without nuclei. On the whole the connective tissue proliferation was more marked in the medulla. Pressure changes were marked. The epithelium lining the bladder and ureters consisted of a narrow band of degenerated cells. The connective tissue beneath was edematous. It had proliferated. Leucocytes and round cells had invaded

the submucous structures. Edema extended throughout the muscular coats. Much of the epithelium of the uterus was necrotic. Leucocytes and round cells were present in large numbers in the submucous structures.

The following cases are more fully illustrative of the condition, since it was possible to diagnose them during life and to verify our clinical findings at autopsy.

No. 1143.—This cow was purchased in the Middle West in December, 1921; bred in November, 1923, and gave birth to a normal calf Aug. 28, 1924. On Nov. 11, about 500 cc. of turbid urine was passed. The later portion of the urine was dark red in color. After considerable straining 10 or 15 cc. of bright blood was evacuated. The blood was not included in the sample collected. The urine was alkaline; specific gravity 1.037; responded strongly to HNO_3, and gave an Esbach reading of 0.25 per cent. There was considerable sediment. It consisted of red cells, leucocytes, and masses of diphtheroid bacilli. Plates prepared from the sediment developed the diphtheroids in overwhelming proportions. On Nov. 12 a blood culture was made. It remained sterile. A physical examination on that day revealed little of note. The temperature was 38.8°C.; pulse 66; respiration 20. No abnormalities were noted in the heart action. The lungs appeared normal. The digestive system appeared to be functioning properly. Rectal examination failed to reveal enlargement of the kidneys. The cow was an extremely high type of dairy animal, the milk secretion averaging about 20 quarts daily. The urine was again examined on Nov. 21. The findings were similar to those recorded except that more albumen was present. On Nov. 26 the cow was slaughtered at an abattoir.

The lesions were confined to the urinary tract. The kidneys appeared normal. The capsule was not adherent. There were no accumulations of urine or other material in the pelvis. The ureters traced from the kidneys to the bladder failed to reveal abnormalities. The bladder appeared as a shrunken, wrinkled, thick walled sac containing only 25 to 30 cc. of blood-stained urine. It measured 12 cm. long by 10.5 cm. wide. The serous surface was congested. The walls were 1.5 cm. thick. The mucosa was swollen. It varied in color from bright yellow to scarlet. Scattered over the ventral surface in the region of the urethra were a few tiny, raised, grayish white patches of exudate. Blood clots partially adherent to the mucosa were also observed. The urethral mucosa appeared normal. The genital tract appeared normal.

The tubes inoculated with bits of kidney cortex and medulla remained sterile. The urine in the bladder when plated contained only diphtheroids. From both horns of the uterus diphtheroids were obtained in small numbers.

Microscopic examination of fixed and stained sections from the kidneys, ureters, uterus, and urethra revealed little abnormal. The lesions in sections from various portions of the bladder varied considerably. Certain areas showed relatively little degeneration of the epithelium. The nuclei of occasional cells were pycnotic.

Leucocytes and round cells had made their way into the mucous coat in small numbers. The submucous coats were edematous and the capillaries engorged. In other portions the mucous membrane was more severely affected. The cells were degenerated or necrotic. Masses of leucocytes were observed in the mucous layer. In circumscribed areas remnants of the epithelial cells were still discernible in dense masses of necrotic red cells, leucocytes, and fibrin. The structures beneath were also invaded by leucocytes. Proliferation of the underlying connective tissue was noted. Bacilli in great numbers were observed in the necrotic material. They were not seen in the deeper structures.

No. 1152.—Grade Holstein cow, purchased in April, 1923. Two normal parturitions, one in June, 1923, and the other in June, 1924.

Jan. 6, 1925. 900 cc. of turbid, deep red urine obtained. Alkaline in reaction; specific gravity 1.025. Tests for albumen were positive (Esbach's, 0.5 per cent). Sugar was not present. On centrifugation, considerable sediment was observed. It was composed of red cells, leucocytes, masses of diphtheroids, and a few cocci. Plate cultures prepared from the sediment developed over 95 per cent colonies of diphtheroids.

Jan. 12. Blood culture negative.

The animal was killed on Jan. 16. The lesions were confined to the urinary tract. The right kidney appeared normal except that one lobule was grayish white in color and tougher than the others. On section the gray color extended through the cortex and into the medulla in the form of fine interlacing lines. One lobe of the left kidney protruded sharply above the others. On palpation it fluctuated. It was composed of a thin wall of cortical tissue, 2 to 3 mm. in thickness, which enclosed clear urine. Both ureters failed to show gross changes throughout their entire length. The bladder was contracted (Fig. 1). The walls were thick. The mucous membrane was swollen and varied from pink to dark red in color, that of the ventral portion was deeply reddened. Patches of variable size of dirty grayish white exudate covered this portion of the mucosa. The exudate when pulled from the mucosa left a raw, reddish gray surface. The mucosa in the more dorsal portions was thrown up into livid, deep or bright red, glistening folds. The urethra presented no abnormalities. The normal uterus contained a fetus.

Stained sections of the kidneys on microscopic examination failed to show abnormalities except for a few small accumulations of round cells. The ureters were normal. The bladder epithelium (Fig. 3) was necrotic throughout two sections. Much of it had disappeared. Small portions were still visible as thin bands of necrotic cells loosely adherent to the underlying structures. The submucous connective tissue was infiltrated with a serous exudate. The connective tissue filaments were forced far apart; their nuclei appeared as tiny round cells situated at wide intervals. The capillaries were packed with red cells. Hemorrhages extending from the capillaries to the bladder surface were not uncommon. In several instances red cells were being discharged into the bladder space. The urethral epithelium was intact. The submucosa was heavily infiltrated with

round cells. The round cell infiltration extended into the muscular structures. The uterus was normal.

Cultures from the cortex of the left kidney remained sterile. Those from the urine within the cyst developed cocci and diphtheroids.

No. 1153.—A grade Holstein cow, purchased Aug. 25, 1924. Calved normally Sept. 5. On Jan. 8, 1925, it was noted that the cow passed turbid urine and at the close of urination bright blood. A little material obtained by swabbing the mucosa about the urethral opening revealed on examination the characteristic bacilli. On Jan. 9, 200 cc. of dark red turbid urine containing blood clots was procured. The reaction was alkaline; specific gravity 1.028; albumen reaction strong, 1.0 per cent; tests for sugar were negative. After centrifugation there was considerable sediment consisting of red cells, blood clots, leucocytes, epithelial cells, and diphtheroids in enormous numbers. Plate cultures developed diphtheroids in practically pure culture. On Jan. 12, blood was obtained for culture. It remained sterile.

On Jan. 16, the cow was slaughtered. The left kidney presented no abnormalities. One lobe of the right kidney was sharply protruded. It was a mottled reddish gray in color, tough in consistency. A calculus was found occluding a large tubule at the tip of a papilla. The pelvic space was increased in size and contained a little yellow exudate. The right ureter appeared normal; the diameter of the left was increased. The bladder (Fig. 2) was contracted and contained 500 cc. of turbid urine. The bladder walls appeared thick. The mucous membrane was swollen and varied in color from yellow to deep red. Patches of grayish white exudate were adherent to the mucosa. They varied in size and general outline. One well isolated round area measured 1.7 cm. in diameter. Its borders were slightly raised above the surrounding epithelium but its center was depressed. In places isolated patches of exudate had become more or less confluent. Attempts to forcibly separate the exudate from the underlying structure left a rough, reddened surface.

Examination of the stained sections of tissues fixed in Zenker's fluid revealed little abnormal in the left kidney. Sections of the mottled lobule of the right kidney revealed a number of wedge-shaped areas rich in round cells. The base of the wedge was located at the surface of the cortex and extended downward a variable distance. A finger-like process of connective tissue, which extended from the capsule between two lobules, could be traced to the thick walled cyst which originally contained the calcareous body. In the papillæ a considerable proliferation of interstitial tissue had occurred. The left ureter failed to show microscopic changes. The right ureter was about twice as thick as the left; the mucous membrane was intact. Small accumulations of round cells were noted just beneath the mucosa. The submucosa was edematous and the capillaries moderately engorged. The bladder lesions were severe. The cells of a considerable portion of the mucous membrane were degenerated or necrotic. Considerable patches of the epithelium had exfoliated so that the bladder surface appeared as a pink-staining, granular mass containing a few leucocytes. Patches of fibrin-

ous exudate, which contained only a few cells, partially covered the underlying structure. The vessels of the submucosa were dilated with red cells and fibrin. The connective tissue of the submucosa had proliferated. It was markedly edematous. The edema extended into the muscular coats. In many of the fixed and stained sections of the bladder it was difficult to find the organism. Apparently it is not highly invasive, since it may readily be found in the urine but has never been encountered in the deeper structures. In a few instances sections of the bladder showing the superficial distribution of the organisms have been obtained. Figs. 4 and 5 are illustrative. Fig. 4 reveals under a low magnification dense masses of bacilli lying in the exudate. Fig. 5 is a higher magnification of the edge of one of the black masses. The characteristic bacilli are plainly visible. The uterus was normal.

Cultures from the cortex of the left kidney and uterus remained sterile.

No. 1154.—A grade Guernsey purchased in the Middle West in 1922; passed through a normal parturition in August, 1923, and in August, 1924. Early in November, 1924, gastrointestinal disturbances were noted. On Nov. 10, the cow attracted attention because of its general poor condition. Urine was first obtained on Nov. 11. It failed to react to tests for albumen. On Dec. 3, 250 cc. of turbid urine was obtained. Its reaction was alkaline; specific gravity 1.037; albumen strong, 1.4 per cent. There were leucocytes, red cells, and diphtheroids in enormous numbers in the sediment (Fig. 6). The bacilli were readily cultivated. On Dec. 27, a blood culture was negative. Examination of the urine on three other occasions gave about the same results. On Jan. 16, 1925, the cow was slaughtered.

The right kidney and ureter were apparently normal. The left kidney (Fig. 7) was mottled grayish pink. Certain lobules were entirely gray, while others contained more or less distinct, irregular gray patches. The gray color in certain lobules extended throughout the cortex and into the medulla. The line of demarcation between the cortex and medulla was indistinct. In two lobules the cortex and medulla were involved to about the same extent. The kidney was tough. The pelvic space was diminished and infiltrated with connective tissue. The walls of the large collecting tubules were greatly thickened. The mucosa of the ureter appeared normal but the walls were thickened. The bladder was contracted and contained only a little blood-tinged urine and several blood clots. The bladder walls were thickened. The swollen mucous membrane was bright red in color and covered with non-adherent purulent mucus.

The stained sections of the right kidney revealed little abnormal except periglomerular and interstitial accumulations of round cells. Those of the right ureter appeared normal. The lesions in the left kidney (Fig. 8) were pronounced. The capsule was thickened and infiltrated with round cells. In places much of the normal cortical structure had disappeared. Occasionally it was possible to recognize the remains of a glomerulus or a tubule. In the main the process consisted of a connective tissue proliferation rich in both small and large round cells. A few of the remaining tubules were filled with leucocytes. The process was less

marked in the medulla, but here there existed irregular accumulations of round cells in areas of proliferating connective tissue. The epithelium of many of the collecting tubules was degenerated and the lumen filled with polymorphonuclear leucocytes. Deep within the medulla the number of tubules was decreased, and the intertubular connective tissue had proliferated and produced marked pressure changes. The walls of the left ureter were thickened, due to edema of the submucous connective tissue. Round cell accumulations were noted in the submucosa. The mucous membrane was intact. The bladder mucosa was more or less degenerated. In places all that remained were a few necrotic epithelial cells sparsely sprinkled over the submucous structures. There was marked edema of the submucous connective tissue. The capillaries were greatly dilated and filled with red cells. Accumulations of round cells were frequent throughout this area. The edema extended into the muscular coats. The mucosa of the urethra was intact but great masses of round cells were present beneath the mucous membrane. Microscopic lesions were not encountered in the sections of the uterus.

Bits of the cortex from both kidneys when implanted into the condensation water of plain agar and blood agar slants remained sterile. Media inoculated from the uterus remained sterile.

No. 1174.—On Jan. 7, 1925, the urine failed to react to tests for albumen and diphtheroids could not be detected in the sediment. On Jan. 23, about 30 cc. of bright blood was passed. This when cultured revealed enormous numbers of diphtheroids. On Jan. 28, turbid, blood-stained urine was obtained. It responded to the albumen tests, 0.5 per cent by Esbach's method. Examination of the sediment revealed fibrin, red cells, leucocytes, and diphtheroids. Urine voided on Jan. 29 was similar in character. Its specific gravity was 1.036. From this time until Feb. 10 the urine was examined at daily intervals. Blood and the bacilli were always present in the sediment. On Feb. 18 the cow was killed.

The right kidney and ureter failed to show abnormalities. In the left kidney one of the large collecting tubules was dilated. It had the appearance of a thick walled cyst filled with clear urine. The left ureter was apparently uninvolved.

The bladder was contracted. The mucous membrane was swollen, bright red in color, and thrown up into broad folds. Several small irregular blood clots were adherent to the mucosa. In other places the membrane was covered with a thick, non-adherent layer of mucopurulent exudate. The urethra was apparently normal. The vagina and uterus presented no gross changes.

Summary of Clinical and Pathological Findings.

From the protocols submitted it will be noted that the cows presented no marked symptoms. The temperature was always within normal limits. Some of the cows were thin but others were in good flesh. A considerable number were admirable dairy types and continued to produce a large quantity of milk until slaughtered.

TABLE I.

The Lesions Encountered in the Urinary Tract of the Thirteen Cases.

No.	Termination.	Bladder.	Ureters.	Kidneys.
1132	Advanced case. Killed.	Severely involved.	Both thickened.	Both severely involved.
1138	" " Died.	" "	" "	" " "
1143	Slaughtered for food.	" "	Not involved.	Not involved.
1151	" " "	" "	" "	Right: calculous; fibrosis of one lobe.
1152	" " "	" "	" "	" fibrosis of several lobes. Left: one lobe cystic.
1153	" " "	" "	Right: thickened. Left: not involved.	Right: calculous; fibrosis of one lobe. Overgrowth of connective tissue in pelvic space. Left: apparently normal.
1154	" " "	" "	Right: " " Left: thickened.	Right: not involved. Left: severely involved.
1172	" " "	" "	Not involved.	Cortex of both sprinkled with tiny round cell areas. No lesions of pelvis.
1173	" " "	" "	" "	Not involved.
1174	" " "	" "	" "	" "
1193	" " "	" "	Both thickened.	" "
1194	" " "	" "	Right: " Left: not involved.	Right: severely involved. Left: fibrosis of several lobes.
1204	" " "	" "	Not obtained.	Pelvis of both invaded by the bacilli.

The most noticeable symptom is the condition of the urine. Attention is frequently drawn to the frequency with which urine is voided. As a rule in the early cases the urine is turbid and the sediment on microscopic examination reveals red cells and leucocytes. Later it is blood-stained and frequently contains blood clots. Usually the cows strain considerably after urination and are apt to pass bright red blood in small quantities.

The lesions may be confined to the bladder, or, in addition, one or both kidneys may be involved. Thickening of the ureter leading from the affected kidney may be pronounced. The bladder is usually contracted. The walls are from 1 to 3 cm. thick. The mucous membrane is edematous and thrown into broad folds. Its color varies from pink to scarlet. In certain cases the mucous layer is covered or replaced by a grayish white, adherent exudate. The affected kidney is enlarged and often firm. It is mottled from gray to red. The pelvic space may be contracted and filled with a thick yellow mass of exudate. The walls of the larger collecting tubules are greatly thickened.

The bacilli are always present in the urinary sediment in large numbers. Suitable preparations from the pelvis of an involved kidney reveal them in equally large numbers.

We have been able to autopsy 13 cases in all. A brief outline of our findings is appended in the table.

It will be noted from the table that our cases may be divided into several groups. The first comprises Nos. 1132, 1138, 1154, and 1194, cows with one or both kidneys severely involved. All revealed a thickening of the walls of one or both ureters. Nos. 1151, 1152, and 1172 all suffered from severe bladder involvement; the kidney lesion was confined to fibrosis of one or more lobules. Whether the fibrosis results from infection with the diphtheroids is not known. It is of interest to note that similar lesions have been encountered by Theobald Smith[8] in young calves following interference with the normal intake of colostrum. It is possible that this lesion had persisted since early life. The ureters failed to show abnormalities. Cystitis was pronounced in Nos. 1153 and 1193, and in both instances the

[8] Smith, T., *J. Exp. Med.*, 1925, xli, 413.

ureters were thickened. The lesions were confined to the bladder in Nos. 1143, 1173, and 1174.

In two instances recovery has been observed. A single case will be cited.

Cow 1203 was known to be infected in November, 1923. A urine examination in January, 1925, confirmed our diagnosis. The animal was slaughtered because of an udder injury in May, 1925. The bladder although contracted presented no abnormality except a considerable increase of submucous connective tissue. The urine was clear and failed to react to tests for albumen. The bacilli could not be cultivated from the sediment.

DISCUSSION.

Among earlier writers considerable discussion has arisen as to whether the infection is an ascending one or whether the organism gains entrance to the kidney from elsewhere within the body.

Certain experimental findings have led several, among them Kitt[9] and Enderlen,[3] to conclude that the disease is a hematogenous infection. Kitt based his assumption that the kidney infection originated by way of the blood stream on the distribution of a miliary type of lesion in both kidneys. He was further impressed by an experiment performed by Enderlen who injected the bacilli into the bladders of rabbits and guinea pigs and failed to note cystitis or nephritis. Intravenous injection of culture into normal rabbits also failed to produce lesions. If, however, a ureter was ligated and the organisms injected into the blood stream, disease resulted in the kidney. Ernst[4] also regards the disease as of hematogenous origin. His material was drawn largely from the abattoirs. In only a few instances were the bladders examined. Of 24 cases examined by him he obtained the tubercle bacillus from 2, *B. coli* from 8, *B. pyogenes* from 12, and diphtheroids from 11. From his study he concluded that pyelonephritis in cattle is largely a hematogenous infection, although in certain cases in which the bladder was first involved the process was regarded as an ascending one. He further pointed out that in vaginitis and metritis following parturition the septic material may gain access to the blood stream and produce a focus within the kidney. The hematogenous origin has been questioned by many, among them Guillebeau and Hess[10] and Ritzenthaler.[11] Ritzenthaler, in an analysis of his series of cases, suggested that the organisms gained access to the bladder by way of the urethra and from there by the process of growth up the ureters, and finally involved the kidneys.

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[9] Kitt, Th., *Path. Anat. Haustiere*, 1901, ii, 471.

[10] Guillebeau and Hess, cited by Ritzenthaler.

[11] Ritzenthaler, M., *J. Comp. Path. and Therap.*, 1910, xxiii, 33.

In the main it seems to us that the several workers have discussed different types of infection and as consequence a generalization or conclusion to cover essentially different processes has resulted. It is also true that in many instances the bladders have not been obtained or that bladder lesions have been overlooked. In kidney disease resulting shortly after parturition, particularly in cases where there has been metritis, the organisms may have gained access to the kidney from the blood stream. The lesion would be largely of the cortex and purulent in character. The organism usually present in bovine metritis, *Bacillus pyogenes*, would be encountered in the kidney. The bladder lesion would be secondary. By contrast, the disease which we have described is relatively specific. We have shown that the kidneys were involved only in certain cases. In a limited number both kidneys were involved, but we have observed involvement in a single kidney in two instances. Evidence exists in certain cases that the pelvis is the first portion of the kidney invaded by the organisms. Here they are found in large numbers, whereas visibly involved portions of the cortex are often sterile when cultured. Involvement of the kidney was always associated with a thickening of the corresponding ureter. In one instance the walls of both ureters were appreciably thickened but the kidneys failed to show lesions. The bladder changes are most characteristic of the disease and occur in all cases. We infer from this evidence that the disease is an ascending infection, the organisms gaining entrance to the bladder and by a process of growth finally reaching the pelvis of the kidney. The organism is not one usually associated with vaginal or uterine infections. In fact, in only a single case did we find uterine lesions. Of the 20 cows from which it was possible to obtain a record, 14 calved normally and 6 aborted.

It was hoped that by means of some biological tests we would be enabled to recognize infections relatively early. With this in view a number of experiments were undertaken. Though only negative results were obtained, mention of some of the more important ones seems advisable. We attempted to ascertain whether agglutinins for the diphtheroid organism were present in greater concentration in the blood of infected animals than in those known to be free from the disease. The titers were about the same in both groups. Introduction of filtered culture fluid into and beneath the skin and on the cor-

nea failed to result in recognizable reactions. At this time we feel justified in asserting that the only specific diagnosis available consists in the microscopic examination of urinary sediment for the characteristic bacilli.

As already stated several have commented on the appearance of the disease after parturition. Of 19 cases in which statistics were available, 7 were recognized during the first 3 months following parturition, 8 occurred from the 4th to the 6th month thereafter, and 4 from the 7th to the 14th month. It is evident that the disease may occur at any time following parturition. However, statistics of such a nature are liable to mislead since the disease is of long standing in many cases and may not be recognized until the processes are well advanced. On the whole the evidence points to specific infection of the urinary tract independent, in the main, of other abnormalities of the genital tract. This view is strengthened by many bacteriological examinations of the exudates from uterine infections, vaginal secretions, etc. Occasionally diphtheroids of similar character may be met with, but usually only in small numbers.

It is obvious that the specific cystitis and pyelonephritis here described may be of considerable economic importance. It is well recognized as the cause of considerable losses among cows in Europe. Such an infection can become well established within a herd before its presence is known. Although the protocols would appear to show that the actual loss is small it must be borne in mind that many of our cases were slaughtered relatively early in the course of the disease. The record of the small herd within which clinical cases were retained shows the existence of a considerable mortality.

SUMMARY.

A specific infection of the urinary tract associated with a diphtheroid has been encountered in cows from three dairy herds. The most characteristic symptom is the frequent passage of turbid or blood-stained urine. The bladder was always found to be involved. The bladder walls were thickened, the mucosa swollen and reddened. Patches of exudate adherent to the bladder mucosa were frequently observed. In certain instances one or both ureters were thickened. In other cases one or both kidneys were involved. The kidney proc-

ess seemed to originate in the pelvis and extend toward the medulla and cortex. The characteristic bacilli were always found in large numbers in the urinary sediment and the pelves of involved kidneys.

EXPLANATION OF PLATES.

PLATE 26.

FIG. 1. Bladder of Cow 1152. About ⅔ actual size. The swollen mucous membrane is congested and thrown up into broad folds. Patches of exudate cover portions of the mucosa.

FIG. 2. Bladder of Cow 1153. About ⅔ actual size. The mucosa appears swollen and congested. Several well defined raised patches of exudate are scattered over the mucosa.

FIG. 3. Section of the bladder of Cow 1152. A portion of loosely adherent necrotic epithelium still remains. Two well defined hemorrhages are visible in the submucosa. The disappearance of the mucous membrane permits the passage of red cells into the urine. Engorgement of the capillaries and edema of the submucous connective tissue are pronounced. Stain, eosin and methylene blue. × 86.

PLATE 27.

FIG. 4. Section of bladder of Cow 1153. The section was stained by the Gram method and counterstained with carmine. The black masses beneath the necrotic exudate are masses of bacilli. × 86.

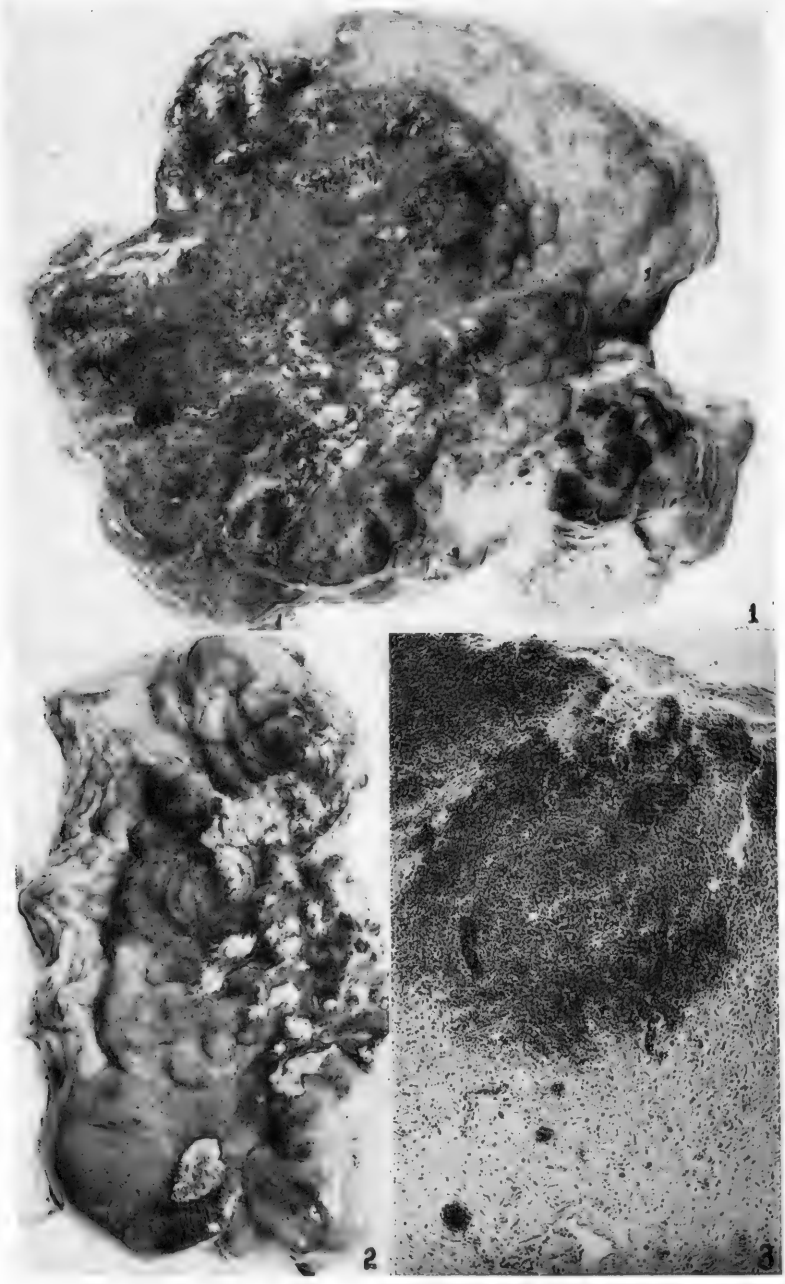
FIG. 5. A higher magnification from the edge of one of the black masses in Fig. 4. Relatively few of the diphtheroids are in focus owing to the thickness and density of the mass. × 810.

FIG. 6. Film from the urinary sediment of Cow 1154. Bacilli are present in considerable numbers. Methylene blue. × 810.

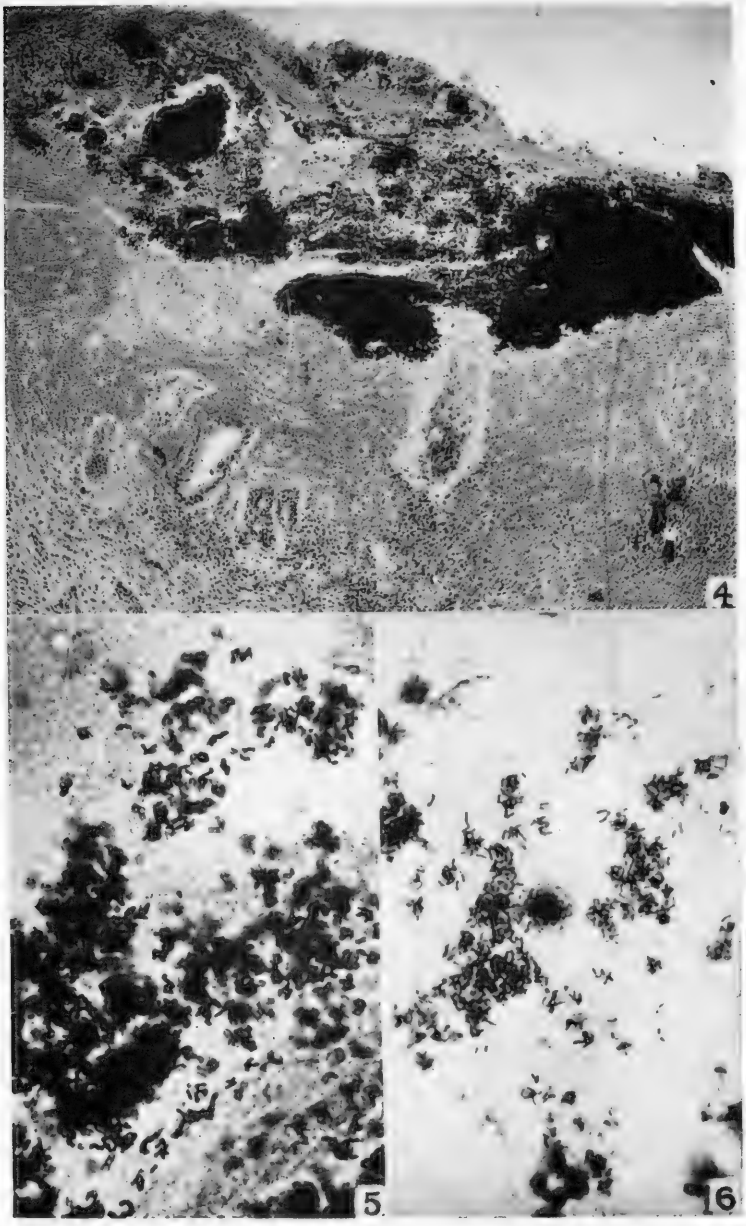
PLATE 28.

FIG. 7. The left kidney of Cow 1154. Most of the cortex appears gray, owing to connective tissue proliferation and round cell infiltration. Note the thickening of the large tubules and the infiltration of the pelvis with connective tissue. About ⅔ actual size.

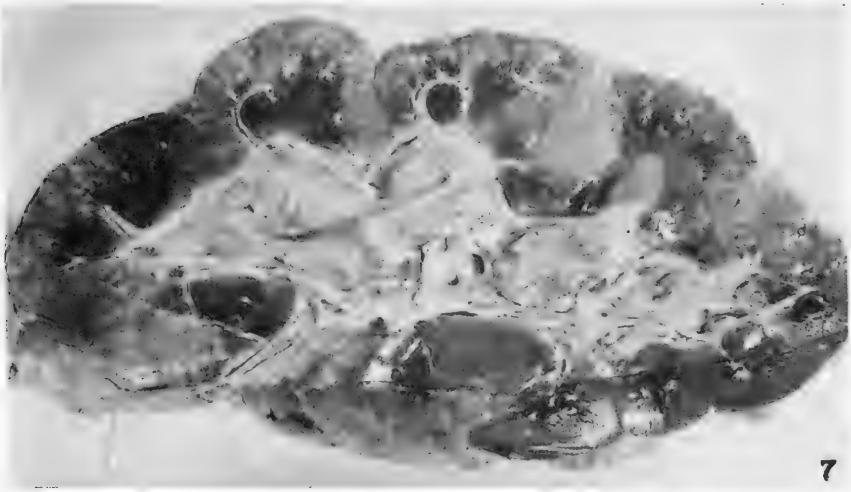
FIG. 8. Section of the cortex of the left kidney of Cow 1154 stained with eosin and methylene blue. A portion of the original structures has been obliterated by connective tissue proliferation and accumulations of round cells. Well defined periglomerular and peritubular accumulations of round cells are illustrated. Two tubules plugged with leucocytes are also visible. × 86.

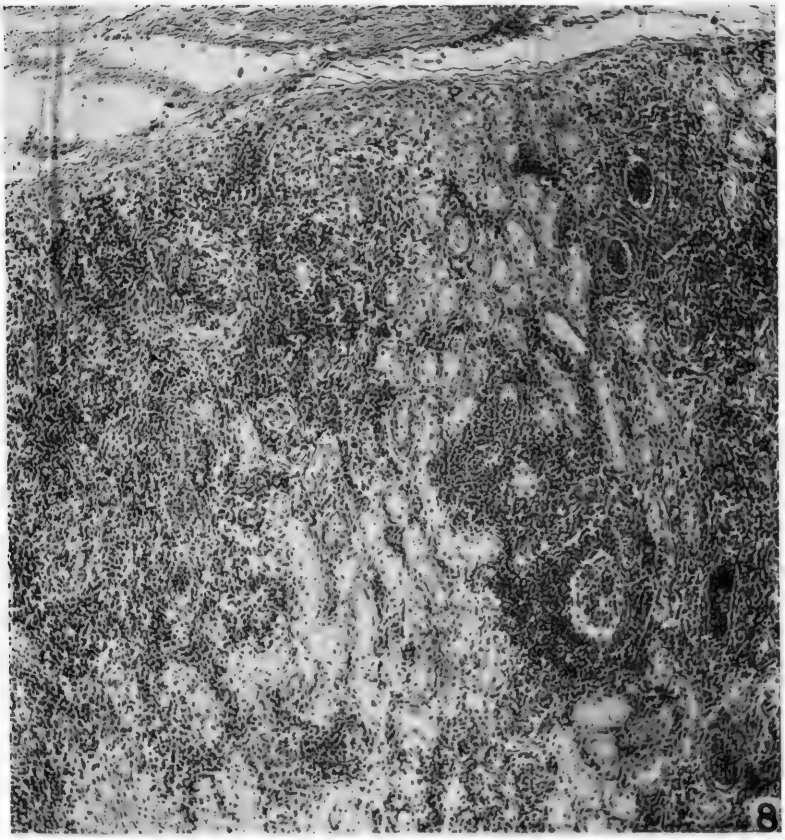


(Jones and Little: Cystitis and pyelonephritis.)



(Jones and Little: Cystitis and pyelonephritis.)





(Jones and Little: Cystitis and pyelonephritis.)

THE EFFECT OF ETHYL URETHANE ANESTHESIA ON THE ACID-BASE EQUILIBRIUM AND CELL CONTENTS OF THE BLOOD.*

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Ethyl urethane has been frequently employed when a prolonged mild anesthesia is desired and it has been found particularly useful for keeping animals quiet during long exposures to x-ray. Our intention had been to use it for this purpose but since the experiments were designed to determine the action of x-ray on the circulating leucocytes and the acid-base equilibrium of the blood it was deemed advisable first to test the influence of the anesthetic itself.

Technique.—The chemical methods used were those devised for determining the acid-base equilibrium of small animals. The carbon dioxide content of the whole blood was determined by the Van Slyke method[1] and the pH by Cullen's method as modified by Hawkins.[2] The relative pH values were corrected and a constant subtracted to obtain absolute pH values at 38°C.[3] During the experiments all the animals were kept under uniform conditions both as to diet and to temperature. 12 hours prior to the observations all food scraps were removed from the cages, and during manipulation the animals were handled carefully to avoid exciting them.

Experiment 1.—20 rabbits were bled from the heart and the CO_2 content and pH of the blood were determined. 16 of the 20 were then given intraperitoneally 0.2 cc., per 100 gm. of animal weight, of a 50 per cent solution of ethyl urethane in sterile physiological saline. This amount was sufficient to produce anesthesia in from 30 to 45 minutes, which lasted from 6 to 7 hours. CO_2 and pH determinations were made at 1 and 3 hours after the injection and 1, 2, and 3 days thereafter. The results are given in Text-fig. 1 and Table I.

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* This investigation was carried out by means of funds from the Rutherford Donation.

[1] Van Slyke, D. D., *Proc. Nat. Acad. Sc.*, 1921, vii, 229.

[2] Hawkins, J. A., *J. Biol. Chem.*, 1923, lvii, 493.

[3] The constants were Rabbit Blood 17, Rat Blood 14.

The pH and CO_2 content of the whole blood of the animals injected with urethane were found to be markedly increased at 1 hour and remained at the high level for 24 hours. The normal level was not reached till 72 hours afterwards. The control animals bled at the same intervals had values within normal limits throughout the experiment.

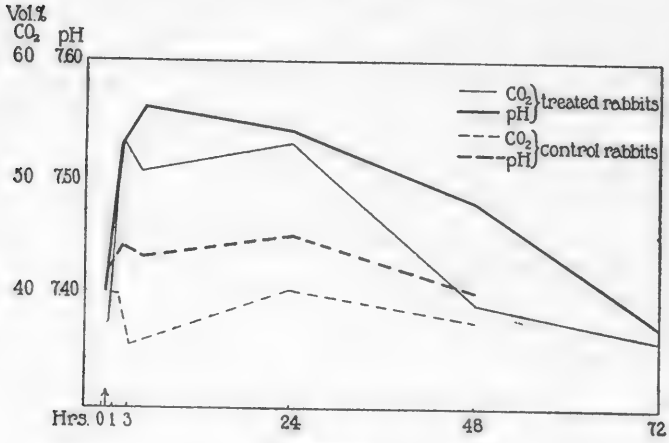
TABLE I.

Effect of Urethane on the pH and CO_2 Content of the Whole Blood of Rabbits.

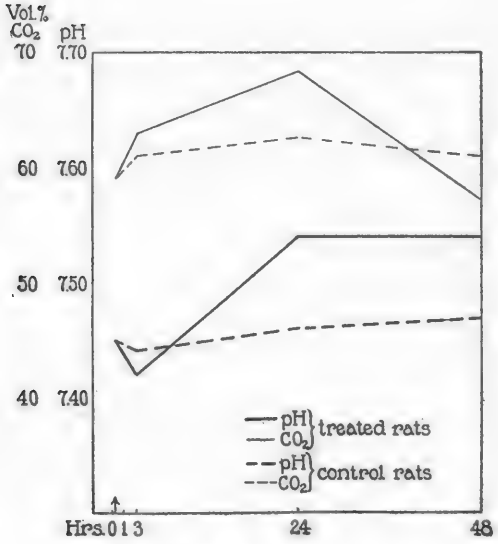
No.	0 hr.		1 hr.		3 hrs.		24 hrs.		48 hrs.		72 hrs.	
	CO_2	pH	CO_2	pH	CO_2	pH	CO_2	pH	CO_2	pH	CO_2	pH
1	35.2	7.30	48.5	7.52			42.0	7.52	39.5	7.53	44.4	7.46
2	29.7	7.19	52.2	7.52			55.2	7.49	28.7	7.27	36.2	7.38
3	39.6	7.39	64.2	7.48			56.8	7.59	38.5	7.45	31.6	7.31
4	34.2	7.34	44.2	7.45			58.8	7.57	35.7	7.41	28.4	7.28
5	44.2	7.43	59.7	7.58			74.8	7.58	43.6	7.52		
6	25.4	7.28	51.1	7.47			58.5	7.50				
7	40.5	7.45	48.6	7.50	39.3	7.62	50.2	7.58			30.2	7.32
8	49.7	7.49	59.1	7.57	71.8	7.62	59.5	7.56			40.3	7.42
9	48.6	7.55	62.8	7.61	61.8	7.59	61.0	7.56				
10	31.8	7.49	39.7	7.59	43.3	7.53	42.2	7.57	47.9	7.57		
11	39.5	7.49	55.5	7.55	44.6	7.53	45.9	7.51	44.9	7.52		
12	32.9	7.48			50.7	7.54			40.6	7.55		
13	39.1	7.46			63.1	7.58	46.2	7.55			42.0	7.51
14	24.8	7.31			36.6	7.51	39.1	7.39				
15	46.9	7.43			54.0	7.55						
16	30.3	7.35			40.7	7.52						
Average...	37.0	7.40	53.2	7.53	50.6	7.56	53.1	7.54	39.9	7.48	36.0	7.37
Controls.												
1	42.4	7.49	38.9	7.50	38.9	7.49	38.4	7.47				
2	41.0	7.40	39.6	7.42	33.5	7.42	42.4	7.42				
3	43.3	7.42	45.6	7.44					37.7	7.44		
4	35.6	7.39	35.4	7.39	34.0	7.38			37.4	7.37		
Average...	40.6	7.42	39.9	7.44	35.4	7.43	40.4	7.45	37.5	7.40		

CO_2 content expressed in volumes per cent.

Experiment 2.—As control the CO_2 content and pH of the whole blood of 10 rats were determined. The specimens were obtained by decapitating the animals and allowing the blood to drip under oil into a small glass cone. The determinations were made by the methods already described. 30 rats were now given intraperitoneally 1 cc., for every 100 gm. weight, of a 10 per cent solution of ethyl urethane



TEXT-FIG. 1.



TEXT-FIG. 2.

in sterile physiological saline. This amount was equivalent to that given to the rabbits and produced an anesthesia in 20 to 30 minutes lasting from 6 to 7 hours. At 3, 24, and 48 hours, respectively, after the injection, 10 treated and 5 normal animals were killed and CO_2 and pH determinations made on their blood. The results are shown in Text-fig. 2 and Table II.

TABLE II.

Effect of Urethane on the pH and CO_2 Content of Rat Blood.

0 hr.		3 hrs.		24 hrs.		48 hrs.	
pH	CO_2	pH	CO_2	pH	CO_2	pH	CO_2
7.49	53.4	7.36	65.8	7.53	73.0	7.42	56.4
7.42	62.1	7.41	55.6	7.52	75.6	7.47	60.4
7.41	61.3	7.38	60.0	7.53	70.4	7.49	58.5
7.44	58.4	7.40	69.3	7.56	76.2	7.49	62.0
7.40	56.7	7.43	63.8	7.51	74.3	7.49	54.2
7.43	65.7	7.41	62.7	7.51	57.9	7.56	54.9
7.51	65.4	7.44	61.0	7.52	59.2	7.55	60.4
7.46	57.3	7.53	58.2	7.51	64.4	7.70	56.2
7.47	58.9	7.36	66.2	7.55	58.3	7.63	61.7
7.47	61.8	7.43	58.8	7.56	58.5	7.64	44.5
Average...7.45	59.1	7.42	62.2	7.53	66.8	7.54	56.9
Controls.		7.41	62.7	7.38	63.8	7.57	59.7
		7.44	61.0	7.47	61.9	7.44	66.0
		7.53	58.2	7.48	63.3	7.51	61.0
		7.36	66.2	7.50	63.7	7.40	56.7
		7.43	58.8	7.46	60.7	7.42	62.1
Average.....		7.44	61.4	7.46	62.7	7.47	61.1

CO_2 content expressed in volumes per cent.

The CO_2 content of the whole blood of the animals injected with urethane was found to be increased at 3 hours and reached a maximum at 24 hours after injection. The pH was normal at 3 hours but had increased to a high level at 24 hours and remained at this level at 48 hours. The CO_2 content and pH of the whole blood of the control animals remained within the normal limits throughout the experiment.

These experiments show that a definite alkali excess exists in the blood of both rats and rabbits after injections of ethyl urethane. This is in accordance with the results of Cushny and Lieb[4] who have shown

[4] Cushny, A. R., and Lieb, C. C., *J. Pharmacol. and Exp. Therap.*, 1915, vi, 451.

that in rabbits under deep urethane anesthesia the reaction of the respiratory center to the blood gases is so altered that an increase in CO_2 or a decrease in O_2 has less accelerating action than is the case normally, while CO_2 accumulates in the blood since the rate of breathing is not accelerated to remove the excess CO_2. The CO_2 is retained in the course of the adjustment to restore the alkaline reaction to normal.[5]

The Action of Urethane Anesthesia on the Circulating Leucocytes.—In the course of a study of the action of x-ray on the blood, it was observed that destructive doses of this agent bring about a condition of uncompensated alkalosis.[6] When the same degree of alkalosis was induced by the injection of sodium bicarbonate it was accompanied by the same changes in the circulating white cells which characterized the x-ray effect. As an extension of this observation, we have studied the effect of the alkalosis produced by the injection of urethane on the circulating white cells of the blood.

Experiment 3.—Blood counts were made on 15 normal rabbits and 10 of these were then given a dose of urethane in the same amounts as in the previous experiments. Counts were made on all the animals at 1 and 3 hours after the injections and repeated at daily intervals for a week. The results are given in Text-fig. 3 and Table III.

The average of the counts on the ten treated animals showed a decrease of about 20 per cent in the lymphocytes during the 1st hour, a still greater decrease at the end of the 3rd hour, and a maximum decrease of 60 per cent at the end of the 48 hour period. Thereafter the count slowly returned to the normal level, reaching it by the end of 1 week. On the other hand, the polymorphonuclear leucocytes increased rapidly after the urethane injection, reaching a maximum of 200 per cent above normal within 24 hours and thereafter slowly returning to normal.

Experiment 4.—Blood counts were made on 39 rats on 2 consecutive days, twenty-six of these being given urethane in the same amount as in Experiment 2. Counts were made on all the animals at 3 hours and at 1, 2, and 6 days after the injection of the urethane. The results are given in Table IV and Text-fig. 4.

[5] Van Slyke, D. D., *J. Biol. Chem.*, 1921, xlviii, 153.
[6] Hussey, R. G., *J. Gen. Physiol.*, 1922, iv, 511.

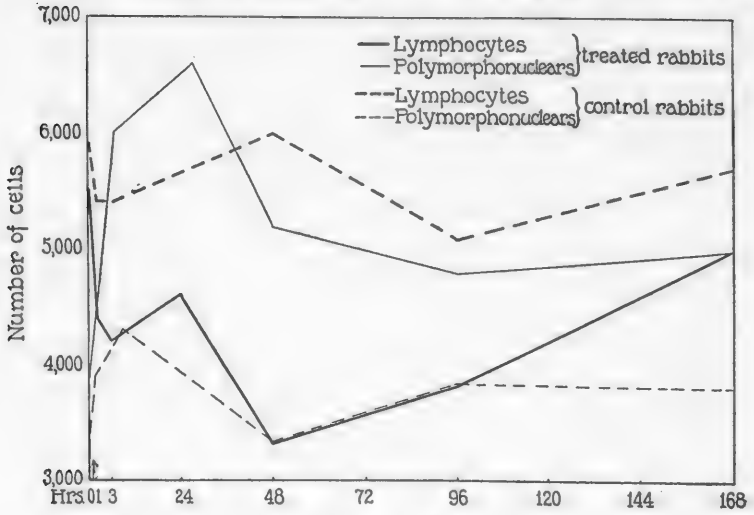
TABLE III.

Effect of Urethane on the Leucocytes of the Blood of Rabbits.

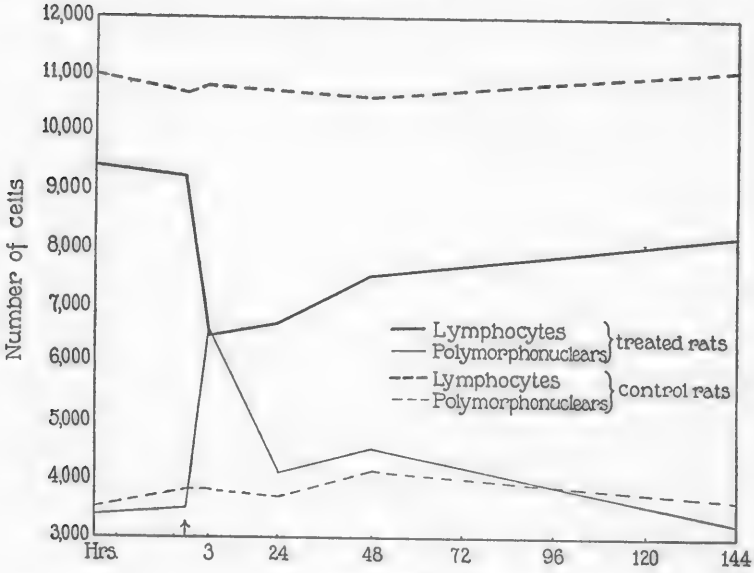
No.	0 hr.		1 hr.		3 hrs.		24 hrs.		48 hrs.		96 hrs.		168 hrs.	
	L.*	P.†	L.	P.	L.	P.	L.	P.	L.	P.	L.	P.	L.	P.
	2,000	3,100	2,500	4,400	1,600	4,800	2,200	4,900	1,200	3,800	2,700	7,200	3,500	4,600
2	8,100	4,500	5,900	4,500	4,600	4,100	3,000	1,400	2,200	2,500	4,600	2,800	8,700	6,100
3	4,700	4,300	4,300	5,200	3,700	5,500	3,500	4,800	2,600	3,700	2,300	6,500	3,700	5,100
4	6,100	2,400	4,800	3,700	4,000	3,500	3,600	5,900	2,100	3,500	5,300	2,800	3,900	4,200
5	3,000	5,500	2,900	6,400	2,600	7,800	2,400	10,000	3,600	4,900				
6	5,800	3,400	2,400	2,800	4,000	7,600	9,300	10,700	4,800	6,100				
7	5,200	4,000	3,800	5,800	5,100	6,100	1,300	11,100	4,800	8,500				
8	7,200	3,400	5,200	4,100	3,900	5,800	6,800	5,500	3,400	7,500				
9	7,300	2,900	9,000	7,000	8,900	9,400	7,300	5,300	4,500	4,600				
10	5,700	5,000	3,300	5,900	4,900	5,100	6,800	6,200	3,800	6,700				
Average..	5,500	3,900	4,400	5,000	4,200	6,000	4,600	6,600	3,300	5,200	3,800	4,800	5,000	5,000
Controls.														
1	6,600	3,200			6,200	5,000			6,500	3,800			6,800	2,900
2	7,000	2,500			6,800	3,600			7,100	2,800			7,600	3,500
3	4,200	4,100			4,000	5,100			4,300	3,600	4,500	4,300	4,500	3,000
4	5,700	2,600	5,000	3,600	4,800	3,600			5,700	3,000	4,900	2,100	4,800	4,000
5	5,800	4,500	5,800	4,300			6,500	3,800	6,600	3,400	5,900	5,100	5,000	5,800
Average..	5,900	3,400	5,400	3,900	5,400	4,300	6,500	3,800	6,000	3,300	5,100	3,800	5,700	3,800

* Lymphocytes.

† Polymorphonuclear leucocytes.



TEXT-FIG. 3.



TEXT-FIG. 4.

TABLE IV.

Effect of Urethane on the Leucocytes of the Blood of Rats.

No.	0 hr.		0 hr.		3 hrs.		24 hrs.		48 hrs.		144 hrs.	
	L.	P.	L.	P.	L.	P.	L.	P.	L.	P.	L.	P.
1	9,400	3,000	9,900	3,100	6,300	8,000	7,000	3,700	9,000	3,400	9,600	2,800
2	12,800	3,700	11,900	2,700	6,900	7,300	7,100	3,600	9,200	3,700	9,600	3,000
3	9,900	3,100	11,600	2,800	5,500	7,900	6,700	3,400	8,100	3,000	9,000	2,600
4	12,300	3,300	11,800	3,300	6,600	9,500	7,200	3,100	8,200	2,800	9,000	2,900
5	12,400	1,600	12,800	1,600	6,300	8,700	7,600	3,500	9,200	3,200	8,700	2,800
6	13,000	2,600	13,200	2,900	5,600	9,900	7,300	4,000	9,100	3,400	9,900	2,700
7	11,000	2,700	11,800	2,700	6,400	9,200	9,800	4,800	8,600	5,500	11,100	3,200
8	11,800	1,800	12,600	2,000	7,500	8,200	7,300	3,400	9,000	2,700	9,400	2,400
9	12,500	3,100	12,200	2,800	6,800	8,600	7,300	3,200	9,500	2,900	10,700	1,800
10	12,800	2,500	12,500	2,600	7,700	8,100	7,000	3,200	8,800	2,400	9,700	2,100
11	5,200	1,900	5,100	1,700	6,100	4,300	8,100	5,400	7,800	4,000	6,200	2,700
12	8,100	3,100	7,400	2,900	7,100	4,900	5,500	3,900	6,400	3,000	4,800	2,800
13	7,400	3,500	7,800	2,500	3,200	7,100	6,400	3,800	6,000	4,500	7,100	3,500
14	9,100	3,100	9,000	2,300	9,400	3,100	7,300	4,000	7,500	4,800	10,000	2,400
15	7,400	4,000	7,800	3,100	5,200	4,500	5,400	3,400	5,200	7,400	7,000	1,900
16	8,300	2,900	7,400	2,700	5,400	3,900	5,600	4,900	5,200	2,900	7,000	2,000
17	11,900	2,800	11,400	5,000	9,000	7,400	9,900	5,900	10,200	6,000	12,900	4,000
18	8,600	3,500	7,800	3,700	5,600	5,600	7,400	4,400	7,000	5,800	8,600	2,600
19	9,700	3,400	8,600	3,200	6,400	6,600	5,800	2,400	6,600	3,300	9,400	2,800
20	10,000	4,200	10,600	6,200	5,800	8,400	7,200	3,800	7,900	4,800	10,000	5,300
21	7,400	2,700	6,600	2,900	6,000	4,500	5,800	4,600	9,200	4,800	7,400	3,800
22	6,900	2,900	7,300	2,600	5,800	5,400	4,400	4,000	6,400	5,200	5,800	3,800
23	8,600	4,400	9,400	3,400	4,000	6,000	7,400	3,600	8,200	5,200	8,000	3,600

24	10,400	4,400	9,800	4,400	6,600	5,400	5,600	4,600	9,800	4,400	9,000	3,000
25	12,000	5,400	11,000	5,000	6,200	6,000	5,200	5,100	8,800	5,200	9,200	5,400
26	11,500	4,000	11,600	3,200	6,400	5,000	6,300	4,600	6,000	7,500	7,200	5,400
Average....	9,400	3,400	9,200	3,500	6,500	6,600	6,700	4,100	7,500	4,500	8,200	3,200
Controls.												
1	13,200	2,100	13,000	2,300	13,200	1,900	14,000	2,300	13,800	2,200	13,900	2,200
2	13,300	2,000	12,600	2,100	13,300	1,800	14,000	2,300	14,400	2,300	14,000	2,400
3	13,700	2,200	13,800	2,200	12,700	2,000	13,300	2,000	13,500	2,200	13,400	2,200
4	13,400	2,200	14,100	2,400	14,100	2,200	14,000	2,300	14,500	2,100	14,100	2,300
5	11,700	3,700	12,800	3,200	10,700	5,500	12,500	4,600	10,100	4,500	12,700	4,200
6	10,700	3,400	8,900	3,800	9,500	4,000	8,000	4,600	9,100	4,900	12,000	5,100
7	11,300	4,000	10,800	5,300	11,100	6,300	12,300	4,000	9,900	6,000	10,200	7,100
8	12,500	6,200	11,800	6,100	11,800	6,400	11,800	5,000	13,500	5,800	14,000	5,700
9	10,300	5,000	11,200	6,500	14,400	2,700	10,800	4,800	11,400	6,400	13,500	6,100
10	6,700	3,200	8,200	2,000	8,500	2,400	6,900	2,700	7,300	4,300	6,300	2,300
11	6,800	3,500	7,700	3,600	7,100	4,000	7,000	3,000	7,200	2,700	8,400	2,700
12	10,600	3,700	10,800	2,700	10,500	3,100	10,000	2,800	9,700	4,600	8,400	4,300
13	8,500	3,300	8,200	4,200	8,200	3,600	7,300	5,300	8,400	3,300	7,500	3,700
Average....	10,700	3,500	10,700	3,800	10,800	3,800	10,700	3,700	10,600	4,100	11,100	3,600

The average of these counts showed a decrease in the lymphocytes of about 50 per cent in 3 hours after the urethane, which persisted for 48 hours with an approximate return to the normal in 6 days. The polymorphonuclear leucocytes increased rapidly after the injection of urethane reaching a maximum of over 200 per cent at 3 hours and then slowly returned to normal.[7]

The marked and persistent alkalosis of the blood associated with urethane anesthesia eliminates the use of this agent in all experiments designed to study the acid-base equilibrium of the blood.

DISCUSSION.

The blood cell changes accompanying urethane anesthesia are of interest on account of the possible association of alkalosis in general with decreases in the lymphocytes. Work from this laboratory[6] has shown that a dose of x-ray sufficient to destroy the lymphoid tissues brings about a state of uncompensated alkalosis while the same degree of alkalosis when induced by the injection of sodium bicarbonate is accompanied by an identical change in the blood picture. These three observations are suggestive of a possible association between the reaction of the blood and the cell content but the processes involved are so complicated that perhaps even a tentative deduction would be unwarranted. However, the marked effect of urethane anesthesia revealed by the blood counts may explain some of the prevailing differences of opinion as to the action of x-ray on the circulating lymphocytes, since this method of anesthesia has been extensively used by some of the workers experimenting with x-ray exposures.

CONCLUSIONS.

When anesthesia is produced in rabbits or rats by injections of ethyl urethane, the CO_2 content and pH of the whole blood of the animal are more or less rapidly increased to a point where there is a marked uncompensated alkalosis which reaches its maximum at 24 hours and persists for 48 hours.

The increase in the CO_2 content and pH of the whole blood is accompanied by a decrease in the circulating lymphocytes and an increase in the polymorphonuclear leucocytes.

[7] It is of interest to note that, in some preliminary experiments, rats under urethane anesthesia, given an exposure to x-ray well below the lethal dose, invariably died between 7 and 14 days later.

UROBILIN PHYSIOLOGY AND PATHOLOGY.

V. The Relation between Urobilin and Conditions Involving Increased Red Cell Destruction.

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Much of the interest in urobilin has come from the fact that it is found in the urine of patients suffering from diseases characterized by an excessive destruction of red blood cells. The occurrence of urobilinuria has been reported in hemolytic icterus, pernicious anemia, malaria, and under various circumstances involving hemorrhage, as, for example, those of hemophilia, apoplexy, and hemothorax.[1–3] There have been many clinical studies in such connection, but little experimental work has been reported, and despite a general belief in the importance of the phenomenon, no uniform conception has developed of its pathological or diagnostic significance in the conditions mentioned.

Many workers have assumed that urobilin may be manufactured directly from the hemoglobin or bilirubin accumulation in the tissues as result of blood extravasation and destruction. The pigment thus produced is supposed to be carried by the blood to the kidneys and excreted by the urine. And yet Kunkel[4] was unable to show this experimentally; and Quincke[5] repeatedly attempted to demonstrate urobilin in old blood clots without success.

The presence of large amounts of urobilin in the stools of patients suffering from hemolysis has long been recognized. Indeed, the estimation of the pigment in the feces has, in recent years, been taken as an approximate measure of the amount of red cell destruction.[6] Yet, many extraneous factors have been shown

[1] Hoppe-Seyler, G., *Virchows Arch. path. Anat.* 1891, cxxiv, 30.

[2] Meyer-Betz, F., *Ergebn. inn. Med. u. Kinderheilk.*, 1913, xii, 733.

[3] Eppinger, H., Die hepato-lienalen Erkrankungen, p. 76, in Enzyklopædie der klinischen Medizin, Berlin, 1920.

[4] Kunkel, A., *Virchows Arch. path. Anat.*, 1880, lxxix, 455.

[5] Quincke, H., *Virchows Arch. path. Anat.*, 1884, xcv, 125.

[6] Robertson, O. H., *Arch. Int. Med.*, 1915, xv, 1072.

to influence in practice the output of fecal urobilin. Even in healthy animals these cause wide fluctuation in the values.[7]

In our earlier papers the normal physiology of urobilin has been studied.[7–9] The communication[10] immediately preceding the present one dealt with conditions of liver damage and biliary obstruction. We are here concerned with the effects of increased blood destruction and allied conditions. For the purposes of the investigation the quantitative measurement of urobilin was made by a method which has already been described.[8] The errors encountered in the study of bile obtained from the open fistula of Schwann have been avoided by a sterile method of intubation,[7,11] which renders possible precise determinations of the entire liver output.

Methods.

The general methods adopted for the intubation of the bile ducts of dogs, care of the animals, and the daily collection of and pigment determinations on urine, stool, and bile have already been described.[7,8] All the animals were permanently intubated, some for the collection of the total bile, others for that of but a sample of the liver output, while still others were so intubated as to permit the bile to flow either into the duodenum or to the collecting bag at the will of the observer. Every day or two portions of the collected bile were incubated with agar to determine whether it had become infected, and furthermore, centrifugalized specimens were frequently examined to the same end. When bacteria were found the animal was discarded for the purposes of this work. All the experimental procedures were carried out under aseptic conditions.

In some instances the effects of an excessive destruction of red blood cells, consequent on intercurrent disturbances, were studied, while in others destruction was induced by the giving of a hemolytic substance or by the intravenous injection of distilled water. Several instances of localized infection, especially of the respiratory tract, accompanied by increased blood destruction, came under observation.

Toluylenediamine in aqueous solution causes a marked red cell destruction.[12] It was given, through a stomach tube, in doses averaging 0.05 to 0.1 gm. per kilo of animal. Icterus developed, yet the dogs remained active and in what appeared

[7] McMaster, P. D., and Elman, R., *J. Exp. Med.*, 1925, xli, 513.
[8] Elman, R., and McMaster, P. D., *J. Exp. Med.*, 1925, xli, 503.
[9] McMaster, P. D., and Elman, R., *J. Exp. Med.*, 1925, xli, 719.
[10] Elman, R., and McMaster, P. D., *J. Exp. Med.*, 1925, xlii, 99.
[11] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 11.
[12] Eppinger, H., Die hepato-lienalen Erkrankungen, p. 124, Berlin, 1920.

to be good condition. Toluylenediamine not only damages the red cells, however, but causes more or less severe liver injury.[10] Sodium oleate, 50 to 75 cc. of a 1 per cent aqueous solution, was given to some animals intravenously on successive days.

To produce an immediate hemolysis distilled water was employed. It was either given directly into a vein,—in amounts of 250 to 300 cc. of it to animals of about 10 kilos during a period of 3 to 5 minutes,—or 100 to 150 cc. of blood was withdrawn, shaken with 200 to 250 cc. of sterile water to hemolyze it, and the mixture reinjected after straining it through a cotton mesh.

The effects of the extravasation of blood into the tissues could be studied as an incidental result of the intubation, since the operative procedures inevitably involved more or less hemorrhage into the tissues about the incision. In certain cases artificial hematomas were produced by the subcutaneous injection of sterile blood, usually obtained from the animal's own vein.

For special purposes, large amounts of urobilin-free bile (two to three times the ordinary 24 hour output of the animal) were given by gavage. To imitate the increased passage of bilirubin into the intestine which follows upon increased blood destruction this pigment was given by gavage on one occasion. It had been prepared from fresh dog bile by a method already described.[9]

In all of the cases the percentage of the circulating hemoglobin was determined daily by the Newcomer method, following a procedure already described.[7] This percentage varies somewhat from day to day and in the absence of any complication the output of bilirubin in the bile of intubated dogs tends to vary in parallel with it.[13] The relation that exists between the two pigments is not strictly quantitative, yet it is sufficiently close to give ground for the supposition that excessive blood destruction is going on whenever any marked increase occurs in the bilirubin output.[14]

Absence of Urobilinuria during Increased Blood Destruction under Conditions Involving Total Bile Loss.

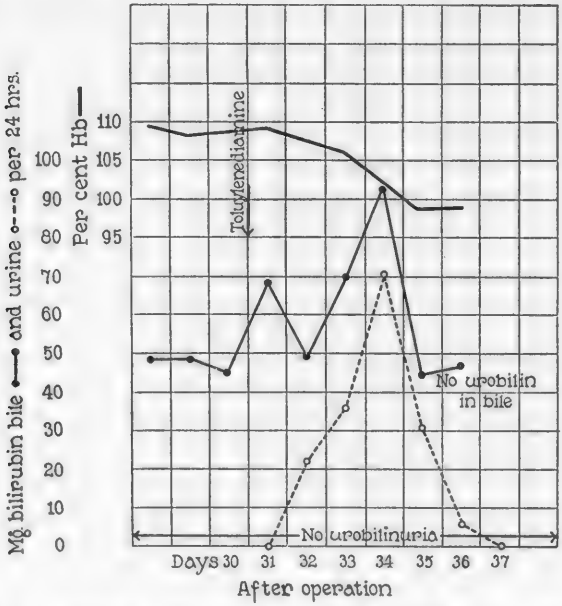
It may be recalled from our previous papers that when the common duct is so intubated that none of the bile reaches the intestine but the whole is lost to the organism, urobilin disappears from this secretion as also from the stools and urine;[7] and even serious damage to the liver does not cause it to reappear.[10] The same holds true when the damage has been to the blood, or to both blood and liver, as in the case of toluylenediamine.

[13] Broun, G. O., McMaster, P. D., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 733.
[14] Rous, P., *Physiol. Rev.*, 1923, iii, 75.

Specimen Protocols.

I. Female dog, weight 9¾ kilos (see Text-fig. 1).

Under ether, intubation of the common duct was performed. By the 3rd day the stool and urine had become urobilin-free, and they remained so thereafter.



TEXT-FIG. 1. Absence of urobilinuria during pathological blood destruction in a dog losing the total bile.

Toluylenediamine in aqueous solution was given by mouth to an animal with intubated common duct. There resulted a progressive fall in the percentage of circulating hemoglobin, and the bilirubin output nearly doubled. There was a marked bilirubinuria and tissue icterus, yet no urobilin was to be found in the bile or urine, and the stools remained consistently acholic and practically free from urobilin (see Protocol I).

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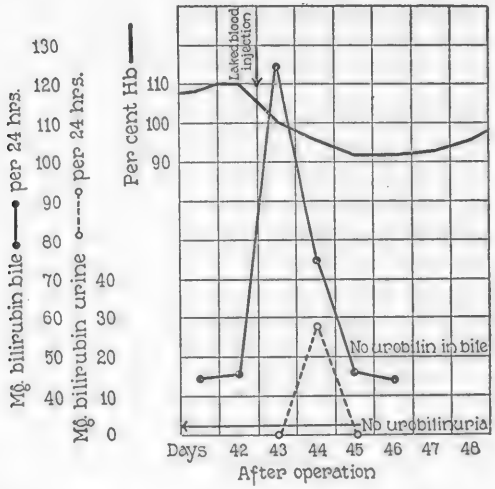
1 month after operation the animal was healthy and active; and the bilirubin output of the bile was constant at about 50 mg. in 24 hours. A small dose (0.475 gm.) of toluylenediamine was now given. There resulted, as the chart shows, a progressive fall in hemoglobin; and a tissue icterus developed which persisted several days. The bilirubin output in the bile reached a maximum of 92 mg. in the fourth 24 hour specimen after administration of the hemolytic agent. Bili-

rubinuria was marked. At no time however was urobilin found in the bile or urine. The stools contained about 2 or 3 mg. in each 24 hours. The presence of this amount is to be attributed to the escape of bile pigment from the jaundiced wall of the gut into the lumen, with subsequent bacterial action.[7]

II. Male dog, weight 10½ kilos (see Text-fig. 2).

The common duct had been cannulated under ether anesthesia, and daily collections of the bile made. It remained sterile, and, after the 3rd day, contained no urobilin.



TEXT-FIG. 2. Absence of urobilinuria in a dog losing the total bile and injected with hemolyzed blood.

A portion of the animal's own blood laked outside of the body with distilled water was injected intravenously. The drop in hemoglobin was 18 per cent, and there was hemoglobinuria and bilirubinuria, yet no urobilin was found at any time in the urine, bile, or feces (see Protocol II).

After 6 weeks collection of bile specimens, 100 cc. of blood was withdrawn from the jugular vein of the animal, shaken with 200 cc. of sterile, distilled water, and the whole reinjected intravenously after straining. The injection required 3 minutes. The bilirubin output rose in the next 24 hours from the "normal" of 45 mg. to 125 mg. The urine contained large amounts of hemoglobin, and, on the day following, much bilirubin. The hemoglobin percentage fell 18 points. In 3 days the bile and urine had returned to normal. At no time was urobilin found in the bile, the urine, or the stools.

Urobilinuria after Increased Blood Destruction under Conditions of Partial Bile Loss.

In the course of our previous work it was shown[7] that when only a fraction of the bile is collected each day, with the rest flowing into the intestine as usual, urobilin continues to be a constituent of both bile and stools, while furthermore, upon liver injury,[10] urobilinuria develops. Sodium oleate exerts a direct hemolytic action on the red cells, even *in vitro*,[15] but there seems to be no evidence that it injures the liver. Following the giving of it urobilinuria is usually not pronounced. Toluylenediamine injures the liver as well as the blood,[12] and it might be expected from what has just been said, that a marked urobilinuria would follow the giving of it, as is the actual case. The amount of urobilin in the stools increases greatly, in corollary to the increase in the amount of bilirubin reaching the bowel as the result of blood destruction.

The difference in the degree of urobilinuria in the two instances selected for illustration may lie in the fact that toluylenediamine, unlike sodium oleate, causes liver damage, but the protracted action of the substance would in itself lead to a sustained and perhaps a cumulation effect. The effect of the intravenous injections of sodium oleate is practically immediate and may be completed within a few minutes. The fall in the hemoglobin percentage was about the same in each of the instances now to be given.

Specimen Protocol.

III. Male dog, weight 10¾ kilos (see Text-fig. 3).

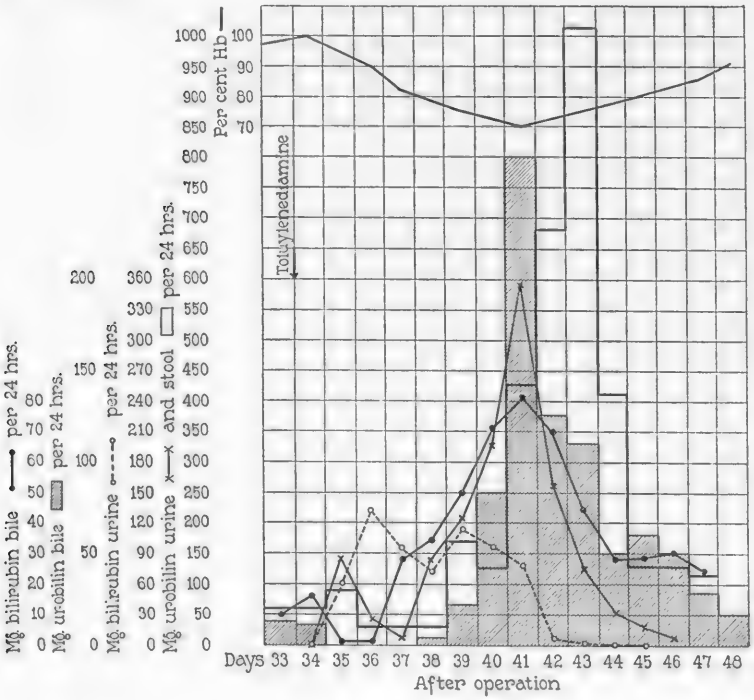
30 days after a cannula had been placed in the left lateral bile duct under ether anesthesia (draining 27 per cent of the liver) the animal received 1.2 gm. of toluylenediamine. The findings for the first 3 days thereafter, when there was a transient, if almost complete, bile suppression, have been discussed previously in another connection.[10] Here we are concerned only with the occurrences after the resumption of the bile flow.

On the 4th day after giving the drug, or the 37th of intubation, the drop in the hemoglobin percentage had become well defined, and it reached its lowest point 3 days later. During this later period of the progressive blood destruction there took place an enormous increase in the output of bilirubin and urobilin in the bile of each day. The polycholia found expression a little later in a great increase in

[15] McPhedran, W. F., *J. Exp. Med.*, 1913, xviii, 527.

the urobilin of the stools. The disturbances subsided gradually, and in 10 days after the giving of the toluylenediamine its effect was almost spent.

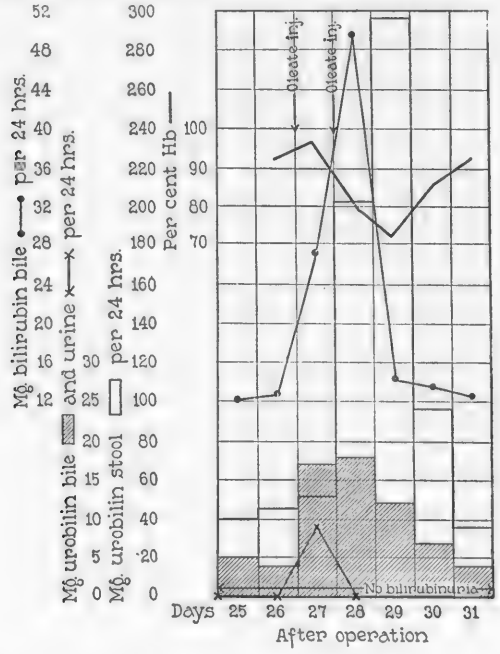
The urobilinuria attendant upon the blood destruction was intense, and in general followed the curve representing the increase in bilirubin elimination in the


Per cent Hb
Toluylenediamine
Mg. bilirubin bile per 24 hrs.
Mg. urobilin bile per 24 hrs.
Mg. bilirubin urine per 24 hrs.
Mg. urobilin urine and stool per 24 hrs.
Days 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48
After operation

TEXT-FIG. 3. Urobilinuria during excessive blood destruction in a dog losing but a fraction of the total bile.

Toluylenediamine in aqueous solution was given by stomach tube. The findings for the first 3 days, when there was almost complete bile suppression, have been discussed before.[10] With the resumption of bile flow, and the excretion of bile pigment in great quantity, the bilirubinuria consequent upon the suppression lessened and disappeared, whereas urobilin appeared in stool and urine, the quantity soon becoming great and only lessening again as the output of bilirubin in the bile lessened. Concomitantly there was a parallel increase and then a decrease in the urobilin of the bile. These changes were attended by a fall in hemoglobin of 30 per cent—another gauge of the degree of blood destroyed (see Protocol III).

bile. Bilirubinuria was somewhat less pronounced and of shorter duration, as would follow from the circumstance that the bile flow was not long impeded. Thus bilirubinuria disappeared the day after the peak of the blood destruction was reached, while urobilinuria continued for 3 more days. Such a relationship, which will be further illustrated in experiments to be described, is characteristic of the two pigments in conditions characterized by excessive hemolysis.


Mg. bilirubin bile ● per 24 hrs.
Mg. urobilin bile and urine × per 24 hrs.
Mg. urobilin stool per 24 hrs.
Per cent Hb
Oleate Inj.
Oleate Inj.
No bilirubinuria
Days 25 26 27 28 29 30 31
After operation

TEXT-FIG. 4. Urobilinuria during excessive blood destruction in a dog losing but a fraction of the total bile.

Intravenous injections of sodium oleate were given on successive days. The blood destruction was reflected in a tremendously increased output of bilirubin in the bile. There was a slight urobilinuria when the disturbance was at its height, but no bilirubin ever was found in the urine (see Protocol IV).

Specimen Protocol.

IV. Male dog, weight 12 kilos (see Text-fig. 4).

The intubation under ether anesthesia made it possible to collect each day the bile from the left lateral lobe of the liver, which was found later at necropsy

to comprise 32 per cent of the organ. The bile remained sterile and showed, like the stool, an approximately uniform content of urobilin.

4 weeks after the operation, 50 cc. of a 1 per cent solution of sodium oleate in water was injected intravenously on 2 successive days. There resulted a drop of 20 points in the hemoglobin percentage. The bile of the 24 hours after the first injection contained four times the usual amounts of bilirubin and urobilin. The stool also contained increased amounts of urobilin. There was a further increase in the pigments after the second injection, but 48 hours later the conditions had returned to the previous "normal." There was a transient urobilinuria but no bilirubinuria.

Urobilinuria during the Extravasation of Blood after Operation.

Immediately after the operation to intubate a common duct the output of bile pigment is greater than at later times, and it is especially prone to be so during the second or third 24 hours of bile collection.[13, 16] It has been pointed out that this is, in part, to be accounted for by the pigment derived from blood extravasated during the operative manipulations. The possibility that the anesthetic causes hepatic injury must also be borne in mind in connection with the urobilin findings now to be described.

When the common duct has been intubated, with result that all of the biliary secretion is lost to the organism, the animal rapidly becomes urobilin-free, there being none of the pigment in bile, urine, or stools,[7] despite the blood extravasation and potential liver injury just referred to. But when, on the other hand, the bile from one portion of the liver only is collected while the rest flows as usual to the gut, there is almost regularly urobilinuria following the intubation. In only one instance out of eighteen has it been absent. It develops at a time when animals with intubated common duct are becoming urobilin-free.

The amount of the pigment often does not exceed 3 to 4 mg. per day during the 2 or 3 days that it is present. But in one instance it was abundant (Dog 4), an instance in which the abdominal incision had become infected and purulent. During 4 days, when the infection was at its height, 175 mg. of urobilin was excreted in the urine and the 24 hour bilirubin content of the bile rose from 70 mg. to 103,

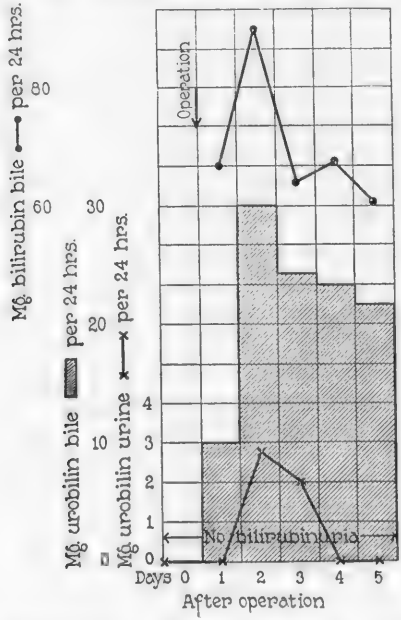
[16] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 395.

107, and 100 mg. on 3 successive days. Even then there was no bilirubinuria. Thereafter the purulence lessened rapidly and the incision healed.

Specimen Protocols.

V. Male dog, weight 12¼ kilos (see Text-fig. 5).

The urine for the 5 days preceding operation was urobilin-free. Intubation under ether anesthesia was performed in such wise that two-thirds of the biliary



TEXT-FIG. 5. Postoperative urobilinuria in a dog losing but a fraction of the total bile.

There was the usual postoperative increase in the bilirubin and urobilin output in the bile. The urobilinuria of the 3rd and 4th days was slight. No bilirubinuria was noted at any time (see Protocol V).

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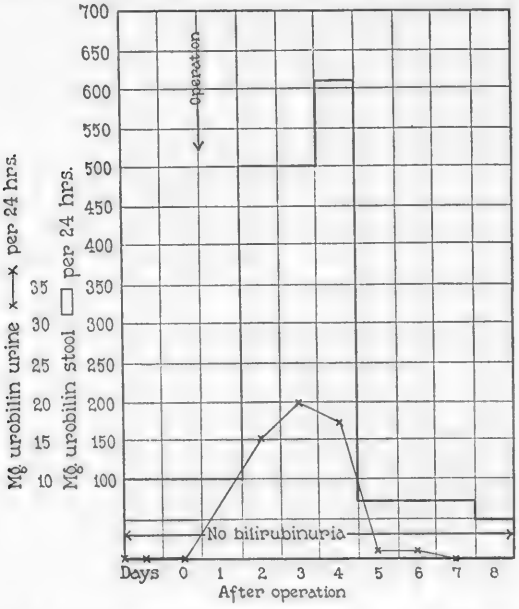
secretion reached the duodenum through the normal channels, while the rest was collected. On the 2nd postoperative day the 24 hour bilirubin content of bile sample rose from 66 to 90 mg. and that of urobilin 10 to 30 mg. The urine during these 2 days showed a mild urobilinuria, and no bilirubin. On the 4th day conditions had returned to normal.

VI. Male dog, weight 12½ kilos (see Text-fig. 6).

Under ether the gall bladder was removed and an "altercursive" intubation was carried out,[7] two cannulas being inserted into the common duct which was cut between. The upper cannula and tube collected all the bile from the liver, while the lower one connected with the duodenum through the ampulla of Vater. The



TEXT-FIG. 6. Urobilinuria following operation in a dog with no bile loss.

An "altercursive" intubation was performed[7] and the total bile allowed to flow into the intestine after operation. The effect of the increased blood destruction, from the absorption of the extravasate due to trauma of the operation, was thus uninfluenced by any bile loss. The urobilin findings resemble those in the preceding experiment, as in it bilirubinuria was absent (see Protocol VI).

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tubes were so arranged that all the bile issuing through the upper one was passed on through the lower into the duodenum, unless some was wished for examination, when it was diverted into a balloon. None was thus diverted during the period now under consideration.

The 24 hour urine specimens, urobilin-free for 3 days preceding the intubation, contained the pigment on the 2nd postoperative day, and for several days thereafter. No urine had been voided on the 1st day. The urobilinuria lasted

longer than usual. No bilirubinuria was found at any time, indicating a complete ability of the liver to handle this pigment. Since no bile was collected, our only measure of the postoperative polycholia was the stool urobilin which was, for a brief period, notably abundant.

Absence of Urobilinuria during the Absorption of Extravasated Blood from Hematomas.

The effects of extravasation of blood into the tissues many days after operation, when there was no suspicion of hepatic injury, were also studied. In seven animals, three draining the total bile, and four, partial bile, artificial hematomas were produced, in the cervical region and in the abdominal walls, by subcutaneous injections of sterile blood, 30 to 60 cc., obtained from the animal's own vein. In all the instances there followed an increase in the bilirubin output through the bile but no urobilinuria.

Urobilinuria Associated with Excessive Blood Destruction during Infections.

Many animals showed evidence of increased blood destruction during infections, as revealed by an increase in the bilirubin output in the bile. These cases are therefore included in this report. One such instance, of infection of a healing incision, has already been referred to, and other similar ones could be cited. In such cases the relative parts played in the causation of the urobilinuria by the operation, the blood extravasation incident thereto, and the infection could not be discriminated.

The most common infection was distemper with which many dogs suffered, particularly during the early spring months. In such of the distemper animals as were losing the total bile urobilin was never found, whereas in those losing but a fraction of it, or none at all ("altercursive" intubation) the affection frequently brought on urobilinuria. The finding has special interest in view of the fact that urobilinuria is encountered during lobar pneumonia in human beings with intact bile ducts.

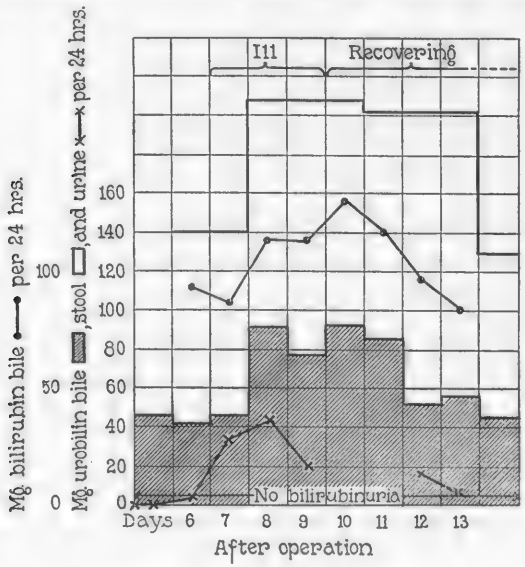
Specimen Protocol.

VII. Female dog, weight 24 kilos (see Text-fig. 7).

6 days after the operation for intubation of one of the branches of the hepatic duct, under ether anesthesia, the animal developed a respiratory infection, be-

ginning with a purulent nasal discharge, and becoming gradually worse until there was frank dyspnea. It was somewhat prostrated, ate very little, and lost its former activity. The severe symptoms lasted only 3 days, and recovery was complete within a week.

On the 2nd day of the animal's illness the amount of bile elaborated by the intubated portion of the liver fell from 97 to 87 cc. but the secretion was darker and



TEXT-FIG. 7. Urobilinuria consequent on respiratory infection in a dog losing but a fraction of the total bile.

The animal had a mild case of distemper characterized by a purulent nasal discharge, a conjunctivitis, and dyspnea. During the disease there was an increase in blood destruction, as shown by the increased output of bilirubin and urobilin in the bile. At the height of the infection there was a mild urobilinuria, but no bilirubinuria (urine specimens on days 10 and 11 were lost and hence are not represented on the chart). The animal recovered in a week (see Protocol VII).

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evidently more concentrated, and there was an absolute increase in the content of bilirubin and urobilin. The biliary changes continued for 3 days after symptoms had disappeared. Urobilinuria of mild degree appeared early, but it diminished and disappeared as the bilirubin output returned to the "normal." There was at no time any bilirubin in the urine. (The urobilinuria figures for days 10 and 11 are not shown on the chart since urine specimens were lost on these days.)

Urobilinuria after the Giving of Large Amounts of Bile by Gavage.

In a preceding paper of the present series[9] the fact has been recorded that when each day there is returned, through a stomach tube, to an animal losing all of the bile after intubation the amount of bile lost, urobilin reappears in the bile and stool, only to disappear again soon after the feedings are stopped. No urobilin appears in the urine as long as the amount of bilirubin given does not exceed the normal pigment output of the liver.

Through inadvertence a dog was one day given a specimen of urobilin-free bile containing two and one-half times the usual amount of bilirubin. To our surprise, the urine next day contained large amounts of urobilin, though none had been found before. The experiment was many times repeated on the same animal, and on others, always with the same result. The urobilinuria was less when the excess of pigment fed was lessened.

Example.—In Dog 25 the giving of 75 mg. of bilirubin per day,—approximately the amount it lost through an intubated common duct,—caused the appearance of urobilin in the bile and stool, but none in the urine. When 100 mg. was administered a slight urobilinuria followed,—5 mg. of the pigment being excreted in 24 hours. When the dose of bile fed was increased so that the dog was receiving 250 mg. of bilirubin per day the 24 hour output of urobilin in the urine was over 100 mg.

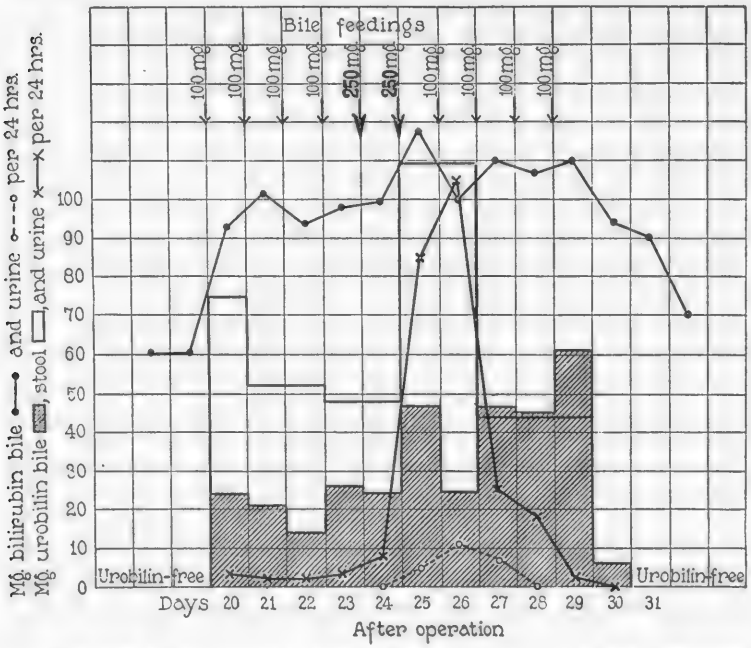
The same experiment was performed on dogs losing only a fraction of their liver output. With them, too, the giving of large amounts of bile by gavage was attended by the development of urobilinuria. In an instance selected for illustration (Protocol IX) the result was attained by feeding a solution of pure crystalline bilirubin prepared from dog bile.[9] We have not sought to learn precisely how considerable an excess of bile must be fed in order to produce urobilinuria. But certainly the excess need not be great. Under the circumstances of the feeding a great deal of bile passes along the intestine at one time—a very different state of affairs from the normal—and doubtless urobilin is formed and absorbed, *en masse*, so to speak, with result that some gets by the liver. In extreme instances, when very great amounts of bile are fed, there may be bilirubinuria as well, an occurrence which Schiff[17] long ago noted.

[17] Schiff, M., *Arch. ges. Physiol.*, 1870, iii, 598.

Specimen Protocols.

VIII. Male dog, weight 16¾ kilos (see Text-fig. 8).

Under ether anesthesia, the common duct was intubated 20 days prior to the experiment. The bile remained sterile, and it contained no urobilin after the 3rd



TEXT-FIG. 8. Urobilinuria and slight bilirubinuria following large bile feedings to a dog losing the total bile.

Bilirubin was returned to the intestine in measured amounts by feeding bile through a stomach tube each day. When the specimen contained 100 mg. of this pigment, urobilin reappeared in the bile and stools, previously free of it. Increasing the amount to 250 mg. for 2 days precipitated a marked urobilinuria, though only traces were present before. It decreased on the resumption of the usual feedings. The stool urobilin showed a parallel rise. Bilirubinuria was slight. 48 hours after feedings had ceased no urobilin was to be found (see Protocol VIII).

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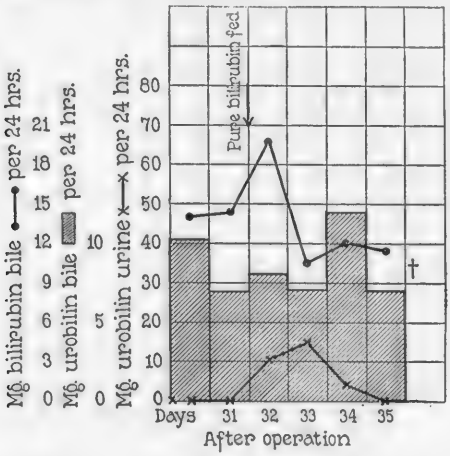
day. On the 20th day specimens of fresh dog bile (liver bile) were fed by stomach tube each day for 10 days. The content of bilirubin was uniform, 100 mg., except on two occasions, the fifth and sixth, when 250 mg. was given. All the specimens fed were urobilin-free.

The first four feedings (each containing 100 mg. of bilirubin—considerably more than the 60 mg. lost) were attended by an increased output of this pigment by the liver, and the appearance of appreciable amounts of urobilin in bile and stool, and of a small quantity (3 to 4 mg. for 24 hours) in the urine. Similar findings in many experiments of this kind have been described in a previous paper.[9] Following the giving of larger amounts of bile, containing over double the amount of bilirubin (250 mg.), the urobilinuria became a striking phenomenon, 205 mg. of the substance appearing in the urine during 72 hours, while there was a slight



TEXT-FIG. 9. Urobilinuria following the feeding of pure bilirubin to a dog losing but a fraction of the total bile.

100 mg. of crystalline dog bilirubin in solution was fed by gavage,—about twice the amount lost by the fractional drainage. The urine, previously urobilin-free, contained, in the next 72 hours, about 30 mg. of this pigment (see Protocol IX).

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bilirubinuria as well. The amount of urobilin fell off as less bile pigment was administered. When the bilirubin fed each day as bile was reduced to 100 mg., the bilirubinuria disappeared, and when the bile feedings were discontinued the animal, within 48 hours, became again entirely urobilin-free, there being no longer any trace of this pigment in the stool or bile.

IX. Male dog, weight 12 kilos (see Text-fig. 9).

32 days after the operation to cannulate a branch of the hepatic duct, under ether anesthesia, at which time the urine was free from bilirubin and urobilin, and the pigment content of the bile sample was uniform from day to day, 100 mg. of pure crystalline bilirubin,—about twice the amount lost daily by the animal,—which had been prepared from fresh dog bile by a method already described,[9] was fed through a stomach tube.

The urine collected 18 hours later contained urobilin and continued to carry it for 48 hours more. Curiously enough, the bile showed no change in its content of this pigment, though it contained more bilirubin than previously. The disturbance was over in 3 days. 5 days after the feeding *B. subtilis* was found in the bile for the first time, doubtless as an air contaminant that had entered the tube system during the manipulations to empty the collecting balloon. The animal was chloroformed at once. No pathological changes were found at autopsy. The cannula was draining 32 per cent of the liver tissue.

DISCUSSION.

The experiments should lead, it would seem, to a better understanding of the relation between urobilin and conditions characterized by excessive blood destruction.

In previous papers[7,10,16] we have proved that normally the formation of urobilin takes place only in the gastrointestinal tract. Even when the liver is seriously injured, or there is biliary obstruction with jaundice, no evidence is to be found of the participation of the tissue cells in general, or of the hepatic cells in particular, in the change of bilirubin to urobilin—so long at least as the liver and bile passages remain sterile. Under such conditions the intestinal source of urobilin remains the only one.

The same fact holds true when there is blood destruction. Extensive hemolysis, sufficient in many instances to lead to tissue icterus, and in some cases associated with liver injury, is never attended by the appearance of urobilin in the bile, urine, or stools, so long as bile is prevented from reaching the duodenum. The extravasation of blood into the tissues does not lead to the excretion of the pigment under such circumstances, nor do infectious processes.

When bile flow to the intestine is going on, as in dogs intubated for the collection of but a fraction of the bile, and blood destruction is produced in one of the ways just mentioned, urobilinuria develops and there is an increase in the urobilin content of bile and stool (see charts). An especially pronounced urobilinuria occurs after toluylenediamine has been given (see Protocol III). During the period of increased blood destruction the curve of urobilin elimination in the urine closely follows both in time and intensity the curves of the increase of bilirubin and urobilin in the bile. Kühl[18] records similar

[18] Kühl, G., *Arch. exp. Path. u. Pharmakol.*, 1924, ciii, 247.

findings after the use of phenylhydrazine. A similar, though less definite relationship was found to exist after intravenous hemolysis (see Protocol IV). In all the cases in which blood destruction occurred or was induced, bile was reaching the intestine through the common duct; in all there was an increase in pigment output by the liver; and in nearly all there was a marked fall in the percentage of the circulating hemoglobin.

What brings about the appearance of urobilin in the urine under such circumstances? Liver damage might be invoked to explain it in some. Though this may, indeed, have been partly responsible in certain instances, it is significant that in every case there was an increase in the urobilin and bilirubin output of the liver, indicating that there can have been no profound impairment in the ability of the organ to excrete the two pigments. During the height of the blood destruction caused by toluylenediamine the liver was, in one instance, excreting eight times the usual amount of bilirubin.

Unexpected light was thrown upon the problem thus posed by the observation that the giving of large amounts of bile by mouth to healthy animals will cause urobilinuria. This happens in animals losing the total bile after intubation, as also in those losing but a fraction of it,—though, the amount of bile which must be fed to produce urobilinuria is larger under the circumstances first mentioned (see Protocols VIII and IX). In such instances, and especially when pure bilirubin has been fed, there can be no question of liver injury. Evidently the determining factor is the presence in the intestine of an unusually large amount of bile pigment. Taken together the facts lead to the conclusion that urobilinuria during increased blood destruction is a secondary manifestation of the polycholia consequent on the liberation of hemoglobin. The large output of bilirubin derived from the liberated blood pigment leads to a large formation of urobilin in the intestines, which is absorbed therefrom in amounts too considerable for all to be dealt with by the liver, with the consequence that part escapes into the general circulation to be removed by the kidneys and urine.

The well known increase in the urobilin content of the stools during the course of diseases involving blood destruction finds a simple exemplification in our observations (see Text-figs. 3, 4, and 6). But it

will be noted that the increase in the stools usually occurs a day or two after the appearance of urobilinuria. Often it is not found at all. As already remarked, the amount of urobilin in the stools is normally subject to great fluctuations. It increases during diarrhea, in the absence of hemolysis,[7] and lessens with constipation. Its value as a measure of increased blood destruction is thus greatly diminished. In our experiments changes in the urobilin quantity in the stool have been far less dependable and significant than the occurrence of urobilin in the urine.

The constant occurrence of urobilinuria during the more acute stages of pernicious anemia has repeatedly excited the attention of clinicians. In such connections the question has often been raised whether there may not be a biliary disturbance traceable to some special involvement of the liver in the disease process. But the ability of the liver of pernicious anemia to excrete large amounts of bilirubin is shown by the finding of tremendous amounts of fecal urobilin in these cases. The responsible factor would seem, from this evidence, to be an augmentation in the rate of red cell destruction, though it is true that van den Bergh,[19] and Broun and others[20] have frequently found hemoglobin, hematin, and bilirubin in the serum of patients with pernicious anemia, and in fatal cases, Peabody and Broun[21] have seen tremendously increased phagocytosis of red cells in the bone marrow removed at autopsy. Warthin,[22] before them, had described similar findings in the spleen, lymph, and hemolymph glands and concluded that "the poison of pernicious anemia stimulates the phagocytes . . . . . to increased hemolysis (cellular hemolysis)." From the evidence of the experiments just discussed blood destruction alone would seem to be sufficient to account for the intense urobilinuria. The passage of increased amounts of bilirubin into the intestine leads to the formation and absorption of more urobilin than the liver, often siderosed and fatty, can handle; the pigment escapes complete removal from the portal stream; reaches the general circulation; and is excreted by the kidneys.

[19] van den Bergh, A. A. H., Der Gallenfarbstoff im Blute, Leiden, 1918.

[20] Broun, G. O., Ames, O., Warren, S., and Peabody, F. W., *J. Clin. Inv.*, 1924–25, i, 295.

[21] Peabody, F. W., and Broun, G. O., *Am. J. Path.*, 1925, i, 169.

[22] Warthin, A. S., *Am. J. Med. Sc.*, 1902, cxxiv, 674.

Urobilinuria has long been known to occur in lobar pneumonia. This, with the occasional icterus, points to a disturbance in pigment metabolism. Since the liver in fatal cases with jaundice and urobilinuria usually shows cloudy swelling and even necrosis, the urobilinuria has been taken by many to be a reflection of the biliary disturbance. But not only is there liver injury to account for the finding, but also some evidence exists of excessive blood destruction. Peabody and Broun[21] have described increased phagocytosis in the bone marrow from patients dying of lobar pneumonia. It may be recalled in this connection that we have found decided increases in the amount of bilirubin put out by the liver of dogs suffering from distemper (see Text-fig. 7). We would suggest that increased blood destruction may well be a factor in the causation of the urobilinuria of infectious diseases though, of course, liver damage must often play a large part.

Thus far the results of frank blood destruction only have been considered. But urobilinuria has also been found in another group of conditions, those characterized by the escape of blood into the tissues with a subsequent slow destruction of the red cells and absorption of the resulting pigments.[2] As already stated we produced hematomas in a few intubated dogs, but the subsequent increases in bilirubin output were very slight, and urobilinuria was not found. On the other hand, this latter phenomenon occurred regularly after operation, in animals from which only a part or none of the bile was being drained. In such of these cases as were intubated there was always to be noted a postoperative increase in the bilirubin of the bile, referable without doubt to the absorption of pigment from the blood extravasate consequent on the trauma of operation. The assumption that the anesthetic caused some slight liver derangement in such cases, a factor lacking when artificial hematomas alone were produced without it, will explain the difference in the urobilin findings.

How may one compare the urobilinuria of excessive blood destruction with that following liver disease? In each instance it is evident that some or all of the pigment has escaped removal from the portal blood by the liver, in the one case because there is too much of it to handle; in the other because the cells are diseased and cannot handle it all. In liver disease the functional reserve is decreased or lost; in excessive blood destruction it is overstepped.

From the practical point of view analysis of the findings reported in this and the preceding paper permits of at least two inferences which may prove to have diagnostic worth. Liver damage as such is never responsible for a high urobilin content of the stool; the amount of this pigment tends, on the contrary, to be low, as would follow from the tendency to bile suppression. In such connection the duration and intensity of the urobilinuria may have considerable value. In acute and severe liver disease urobilin may be abundant in the urine, but only transiently; for it disappears practically as soon as bile ceases to flow into the intestine.[10] The urine thereafter contains only the bilirubin of the developing jaundice.

By contrast with the foregoing when there is sustained blood destruction without serious liver injury urobilinuria is likewise sustained. Bilirubin may also appear in the renal output, if there be secondarily some obstruction to bile outflow, as not infrequently happens on sudden hemolysis. During persistent, sustained blood destruction the urobilinuria varies in degree with the amount of blood destroyed (see Text-fig. 3, which exemplifies this strikingly).

SUMMARY.

Further evidence is presented, in addition to that of our previous papers, that the intestinal tract is, under ordinary circumstances, the sole place of origin of urobilin. So long as the biliary tract remains sterile the presence of the pigment in bile and urine is entirely dependent upon the passage of bile to the intestine.

Animals rendered urobilin-free by the collection of all the bile from the intubated, uninfected common duct, remain urobilin-free during and after extensive blood destruction caused by intravenous injections of distilled water, as also after reinjections of the animal's own blood, hemolyzed *in vitro*. No urobilin appears in the bile, urine, or feces of animals so intubated when blood destruction has been caused by sodium oleate, or by an agent, toluylenediamine, which damages the liver as well as the blood.

On the other hand, when bile flow into the intestine is uninterrupted, urobilinuria occurs during blood destruction caused in any of the ways mentioned and it parallels, both in severity and duration, the destructive process.

Merely increasing the amount of bilirubin within the intestines of healthy dogs by feeding urobilin-free bile, will lead to marked urobilinuria. The extravasation of blood into the tissues, resulting from the trauma of an operation for intubation of a bile duct, does not lead to urobilinuria in animals losing all of the bile after this operation, but may do so when only a small fraction of the bile is drained, while the remainder reaches the intestine as usual. The production of artificial hematomas, without operation, is not followed by urobilinuria, under the circumstances last mentioned, but merely by an increase in the bilirubin of the bile. The effect on the liver of the anesthetic employed during the intubation may be responsible for the difference in the two cases.

During the course of certain intercurrent infections affecting some of the intubated animals, notably distemper, there was a drop in the hemoglobin percentage of the circulating blood, accompanied by an increased output of bile pigment or further by urobilinuria, when the conditions were such that bile still reached the intestine. The findings pointed to increased blood destruction as a factor in the urobilinuria.

The evidence presented, taken with that of our previous papers, suffices to demonstrate, that urobilinuria, occurring during blood destruction, is primarily the result of an increased excretion of bilirubin from which, in turn, an unusually large quantity of urobilin is formed within the intestine. The liver fails to remove from the portal blood all of the latter pigment which is resorbed and consequently some of it reaches the kidneys and urine.

Our work has been carried out on animals with uninfected biliary tracts and livers, save for one case which has special mention. The influence of infection of the biliary tract on the place of formation of urobilin and the development of urobilinuria will be discussed in a succeeding communication.

EXPERIMENTS ON THE GLOMERULAR DISTRIBUTION OF BLOOD IN THE MAMMALIAN KIDNEY.

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Plates 29 and 30.

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Recent observations have shown that in the frog kidney not all of the glomeruli necessarily receive blood, and therefore function, at any one time (1). Conditions were encountered or were experimentally produced which caused the fraction of the total existing number of glomeruli taking part in the function of the whole kidney to vary within wide limits. These conditions related to volume or composition of the blood or to the nervous control of the renal vessels. By these observations an experimental basis has been provided for the conception first presented by Hermann (2)[1] that one part of the excreting surface of the kidney might rest while another part was active. Our understanding of the mode of adjustment of renal function to excretory requirement has been extended to include variability of filtration surface, and the fashion in which the kidney utilizes and preserves its "factors of safety" has in part been revealed. The frog's kidney was chosen for the experiments upon which these statements are based because of its adaptability to direct observation.

Since the method of direct observation of the glomerular circulation has not yet proved successful when applied to the mammalian kidney, less direct methods are required in testing the applicability of these

[1] Khanolkar's (3) ascription of a similar conception to Kobert (Kobert, R., *Arch. exp. Path. u. Pharmakol.*, 1882–83, xvi, 361) appears to be erroneous. Kobert called attention (p. 389) to the unequal distribution of iron in the kidney following intravenous injection of ferric acetate or citrate, and cites the work of Wittich (Wittich, K., *Arch. mikr. Anat.*, 1875, xi, 75) in support of the view that part of the kidney may be active while another part is inactive. Both writers, however, had tubular and not glomerular function in mind.

results to the mammalian organ. Khanolkar (3) has already studied this question in rabbits: hemoglobin was injected intravenously, the kidney promptly excised, and the hemoglobin-containing glomeruli in sections of the cortex counted. His conclusions concerning the "partial activity" of the kidney agree with those drawn by Richards and Schmidt from direct study of the frog's kidney.

The experiments here reported, while similar in design to those of Khanolkar, were carried out by different methods. Intravital staining of the glomeruli by Janus green B has been utilized as a means of identifying those through which blood was flowing at the time of injection, and an expression of the relation which this number bore to the total number of glomeruli in the kidney has been obtained by an adaptation of Nelson's method. Our results are in accord with Hermann's conception and the observations of Richards and Schmidt, and confirm those of Khanolkar.

Nelson's method (4) for enumerating the glomeruli in the mammalian kidney is an application of Bensley's technique for counting the islands of Langerhans in the pancreas (5). It consists in perfusing the vessels of the kidney *in situ* with a solution of Janus green B, followed by a solution of ammonium molybdate. The glomeruli are stained intensely blue, the tubules reddish brown. The cortex is then carefully dissected free from medulla, weighed, and cut into suitable pieces. Several pieces are weighed and the glomeruli in them enumerated. The ratio of weight of the piece in which count has been made to that of the total cortex gives the basis for calculating the number of glomeruli in the cortex.

In one experiment Nelson cut the cortex of a rabbit's kidney into ten pieces and counted the number of glomeruli in each. The total glomerular number was estimated from the count of each piece. When the ten estimates were compared with the actual total obtained by adding the ten counts, it was found that in six estimates the discrepancy between estimated and actual total glomeruli was less than 10 per cent: in the other four it ranged from +19 to −27.7 per cent. It was further shown in three out of four of his experiments that the number of glomeruli in the two kidneys of the same animal is the same within the probable error of the method (greatest difference, 1.3 per cent). Therefore, having found that Janus green B can be used successfully as an intravital glomerular stain by intraaortic injection, the following plan was outlined for determining the number of glomeruli patent to the blood stream under various conditions.

After ligature of its vessels, one kidney (control) was excised from an anesthetized, eviscerated rabbit, perfused with Janus green B, and laid aside for subsequent estimate of the number of glomeruli in it, precisely according to Nelson's directions. The desired experimental condition was then established in the animal,

Janus green B injected into the aorta, and the circulation through the remaining kidney stopped by clamping the aorta within 10 seconds after the end of the injection. Its vessels were perfused with ammonium molybdate and the number of stained glomeruli in its cortex estimated by Nelson's method. The ratio of this figure to the total number of glomeruli estimated in the control kidney was taken to represent the fraction of the total glomeruli through which blood was flowing at the time of injection of dye.

The three chief items in this plan need further comment.

1. Nelson's ten estimates of the total number of glomeruli in a rabbit's kidney, based on counts of the ten sections into which the whole cortex was cut, varied from the actual number by from +19 to −27.7 per cent. Six, however, were within 9 per cent. We have repeated this experiment.[2] The cortex of a kidney weighing 3.24 gm. was cut into sixteen weighed pieces. The glomeruli in each were counted. The estimates based on the counts of eleven of the pieces were within 10 per cent of the actual total number; three were within 15 per cent, and two within 20 per cent.

Using the data of these two experiments it is seen that the probability of selecting a single piece out of the ten or sixteen into which the kidneys were cut, estimate from which should be correct within 10 per cent, is of the order of 60 per cent. If, however, enough pieces are counted to yield three estimates which agree within 10 per cent of their mean, the probability that this is correct within 10 per cent is of the order of 120:1 and 560:1 respectively. Most of the estimates recorded in the accompanying tables were obtained by averaging three estimates which agreed among themselves within 10 per cent of their mean; in the highly constricted kidneys such agreement was not encountered, hence from four to six pieces of kidney were counted.

2. It is impossible to count accurately the unstained glomeruli in a slice of cortex taken from an intravitally stained kidney; hence the decision to use the estimated total number of glomeruli in one kidney as the figure with which to compare the number of intravitally stained glomeruli in the other. Justification of this way of obtaining an expression of the fraction of the glomeruli reached by the dye requires proof that the total number of glomeruli in the two kidneys of the

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[2] The data of this experiment are as follows: Weight of cortex = 3.24 gm.; weights of pieces into which cortex was cut, 0.132, 0.203, 0.233, 0.282, 0.195, 0.204, 0.288, 0.136, 0.196, 0.159, 0.229, 0.128, 0.237, 0.215, 0.239, 0.164 gm.

Glomeruli in each piece = 4899, 7714, 8871, 9869, 6225, 5514, 10,078, 4386, 6888, 5487, 6093, 4445, 7690, 6823, 7927, 5703; total = 108,612.

Estimated total glomeruli from count of each piece = 120,025, 122,653, 123,307, 113,494, 103,335, 87,121, 112,774, 104,386, 113,900, 111,386, 85,911, 112,459, 104,584, 102,345, 107,015, 112,349; average of sixteen estimates, 108,568.

Percentage errors of individual estimates: +10.5, +12.9, +13.6, +4.5, −4.8, −19.8, +3.8, −4.4, +4.9, +2.6, −20.8, +3.5, −3.6, −5.7, −1.0, +3.5.

same animal is the same. As has been stated, this was the case in three out of four of the rabbits studied by Nelson. We have made three experiments specifically designed to test this point, and in all three the glomerular estimate of the two kidneys was the same within less than 10 per cent (8.1, 4.7, 2.8).[3]

As a precaution, however, against gross error, which, in exceptional cases, might arise from marked difference between the two kidneys, we have cut frozen sections from the intravitally stained kidneys in the majority of our experiments and have counted the stained and unstained in a total of 500 glomeruli. The results of this method, which is essentially the same as that used by Khanolkar, are in general remarkably concordant with those obtained by the other. This concordance, which is particularly marked in all of those experiments in which over 90 per cent of the glomeruli in the intravitally stained kidney were stained, can be accepted as additional evidence that as a rule in healthy rabbits the two kidneys contain approximately the same number of glomeruli.

3. The dye Janus green B possesses definite physiological action in which constriction of renal vessels is included. Hence, question arises as to the possible extent to which the renal conditions intentionally established may have been distorted by the reagent used to reveal them.

The first effect in our experiments which resulted from the injection of this dye into the aorta was abrupt decrease in kidney volume, obviously due to peripheral constricting action on renal blood vessels. This is the only action which need concern us here, for by the time the injected dye reaches other organs, action on which might indirectly affect the renal circulation, the glomerular staining is accomplished. That the number of glomeruli actually reached and stained by Janus green B is not less than that which it would have reached were it devoid of constrictor action is shown by the fact that the greatest degree of shrinkage of the kidney was exhibited in those whose vessels were dilated as a result of the action of caffeine or NaCl; yet it is in precisely these experiments that figures show all or nearly all of the glomeruli to have been stained (see Fig. 1). On the other hand, in some of the experiments in which the renal vessels were constricted by the action of adrenalin, injected before the introduction of the dye, very little further constriction by the dye occurred.

In two experiments, we were successful in preventing shrinkage of the kidney by the dye, in one instance by almost simultaneous injection of blood into the aorta *via* the central stump of the superior mesenteric artery; in the other, by simultaneous injection of a carefully chosen dose of caffeine. In neither instance did the result differ widely from those of other similar experiments in which no such precautionary measures were adopted (see Figs. 2 and 3).

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[3] The data of these experiments are as follows: Experiment 1. Glomeruli in right kidney, 178,179; in left kidney, 169,955; difference, 4.7 per cent. Experiment 2. Glomeruli in right kidney, 192,945; in left kidney, 177,240; difference, 8.1 per cent. Experiment 3. Glomeruli in right kidney, 177,544; in left kidney, 182,682; difference, 2.8 per cent.

It might be expected that the introduction of a constrictor substance like Janus green B into the renal vessels would result in such immediate closure of afferent arterioles that onward passage of blood containing it into the glomerular tufts would be arrested and glomerular staining prevented. The facts mentioned above disprove this and direct observation of the reaction of the vessels of the frog's kidney to the presence of a constricting substance in the blood shows the reason. The vessels contract in such fashion that first there is retardation in the normal blood flow, then slow flow of apparently dilute blood, and finally the oozing of clear plasma through the glomerular vessels. Only in instances in which the initial glomerular blood flow was very sluggish did abrupt cessation of the current of fluid in the vessels occur. In such instances, assuming Janus green B to be the constrictor substance studied, the expected result would be staining of the afferent vessel without staining of the glomerular tuft. In a few preparations this result has been encountered but not with sufficient frequency to affect the estimates.

In a few experiments designed to control the results obtained with Janus green B we have substituted India ink[4] as the vital dye. The results of satisfactory experiments obtained with it confirm those obtained with Janus green B.

Technique.

Rabbits used exclusively; preliminary narcosis with urethane by stomach (2 gm. per kilo); ether during operation when necessary. Tracheal cannula. Blood pressure recorded from carotid artery by mercury manometer. Gastrointestinal tract excised after ligature of its vessels. One kidney was removed for total glomerular count, according to Nelson's method. Urine flow from the remaining kidney was measured by a drop recorder, connected with the ureter. In a number of experiments an oncometer was placed on this kidney for recording changes in its volume during the experiment and during the injection of the dye. The abdominal aorta, distal to the renal arteries, was tied, clamped, and a cannula inserted pointing toward the heart.

After the desired circulatory condition had been satisfactorily established, 0.1 to 0.75 cc. of a solution of Janus green B in 0.9 per cent NaCl was injected into the aorta either by a needle thrust through its wall, or by a cannula in the central stump of the superior mesenteric artery. As soon as possible after the end of the injection, circulation through the kidney was stopped by clamping the aorta. The renal vessels were immediately perfused, first with 15 cc. of warm 0.9 per cent

[4] India ink was not used in the majority of experiments for the reasons that in our experience glomerular staining by it is not uniformly accomplished, and its accumulation in tubular vessels is apt to obscure the glomeruli, thus making glomerular count more difficult.

NaCl to wash out blood, then with 5 per cent ammonium molybdate, through the cannula in the aorta.

Both kidneys were decapsulated, weighed,[5] cut, and counted, according to Nelson's technique. Any glomerulus containing a detectible amount of dye was counted as stained.

In experiments numbered below 28, a 1.5 per cent solution of Janus green B[6] in 0.9 per cent NaCl was used: in the rest, a saturated solution in saline was used. This is somewhat less than 3 per cent. 0.75 cc. of this solution injected into the aorta stained glomeruli and afferent vessels intensely; 0.1 cc. stained them sufficiently to allow counting; 0.02 cc. did not stain glomeruli. Glomeruli are not stained by intravenous injection of Janus green B. A mixture of dye and blood loses its ability to stain glomeruli after standing a few minutes.

Several experiments were abandoned because of gross pathological change in the kidneys. The presence of a few small depressed scars on the surface was not considered sufficient abnormality to warrant giving up the experiment. Histological sections of the kidneys of eight animals (50 to 57) showed no glomerular or vascular change.[7] Two (55 and 56) presented small areas of low grade interstitial nephritis. Since in one of these a diuretic caused practically all the glomeruli to be stained, and in the other injection of adrenalin led to a low count, it seemed evident that pathological change of this type, common in rabbits, did not influence the vascular reactions of the kidney.

Distribution of stain within individual glomeruli varied greatly. In diuretic kidneys, the great majority of the tufts were stained a uniform, deep blue. In constricted kidneys many glomeruli showed dye only in a spot of intense blue the diameter of which was about one-eighth that of the tuft, located near the entrance of the afferent vessel. In other glomeruli, the stain was intense in half of the tuft, fading toward the free border; in others only certain capillaries were stained. The glomeruli in the control (perfused) kidney were stained a uniform deep blue, the tubules a pale reddish brown. Frozen sections were cut from two blocks of the control kidney to check the completeness of the staining. In most instances, the number of unstained glomeruli encountered was less than 1 per cent.

[5] Weights of control kidneys are of little value because of different degrees of edema produced during perfusion. Intravitally stained kidneys, perfused with less fluid acquired less edema; hence, weights are more nearly correct.

[6] In accordance with the recommendation of Bensley, the Janus green B made by H. A. Metz and Co. was used in these experiments.

[7] We are indebted to Dr. Morton McCutcheon for examination of these sections.

Control Experiments (Table I).

In order to gain information concerning the number of glomeruli open to the circulation after preparation of the animal as described, nine control experiments were made in which no effort was made to

TABLE I.

Estimations of Glomeruli Open to the Circulation under Control Conditions.

Experiment No.	Sex.	Weight.	Blood pressure.	Urine flow.	Glomeruli stained.		Percentage of stained glomeruli in vitally stained kidney.		Remarks.
					In control kidney.	In vitally stained kidney.	From counts.	From frozen sections.	
		kg.	*mm. Hg*	*drops per min.*					
15	F.	2.0	44	—	108,612 (T)	87,495 (T)	80	—	
11	M.	2.0	60	—	135,753 (4)	80,748 (T)	59	—	Oncometer.
18	F.	1.9	76	—	204,176 (4)	114,824 (5)	56	55	
31	"	2.1	72	5	218,201 (3)	195,370 (3)	89	93	
50	"	3.0	84	6	221,194 (4)	170,833 (3)	77	85	Oncometer and renal blood flow.
51	"	1.8	78	3	189,666 (3)	191,405 (4)	100	96	" "
52	"	1.7	70	1	226,618 (3)	205,248 (3)	91	91	" "
54	"	1.6	82	0.6	184,826 (3)	77,733 (3)	42	31	" "
35	"	3.1	61	2.5	182,108 (3)	119,359 (2)	65	72	Not eviscerated.
43	"	1.4	75	8	144,258 (3)	111,608 (3)	76	79	" "
37	M.	1.3	88	7	241,621 (3)	213,490 (3)	88	94	India ink used instead of Janus green B.
39	"	1.8	100	11	117,693 (3)	98,813 (5)	84	86	" "
69	F.	2.7	86	12	238,352 (3)	162,646 (3)	68	74	Fall in kidney volume prevented by injection of blood.

Figure in brackets following the number of glomeruli indicates the number of pieces of cortex counted. (T) indicates a complete count of the whole kidney.

alter the renal circulation. In these, the number of intravitally stained glomeruli in the left kidney varied from 42 to 100 per cent of the total number in the right. In six, however, the range is from 56 to 89 per cent. These six include one (69) in which decrease in kidney volume was prevented by following the dye injection immediately with an injection of whole blood, kept fluid by heparin, from another rabbit.

In addition, two control experiments are included, in which India ink, injected into the aorta, was used instead of Janus green B, and two others in which the animals were not eviscerated.

In these a small incision was made through the abdominal wall, over the symphysis, for the purpose of permitting the insertion of a cannula into the right ureter. The solution of Janus green B was injected into the aorta through a slender glass tube, attached to a syringe, the tip of which was inserted into the left carotid artery and pushed downward in this until it projected into the aorta. Immediately after injection of the dye the abdomen was opened, the aorta clamped above the renal arteries, and the kidneys perfused with warm salt solution, introduced by a large needle thrust through the aortic wall below the renal vessels. Before injecting the molybdate solution *via* the aorta, the left renal artery was clamped. When the molybdate injection was finished, the left kidney was excised, its vessels perfused with Janus green B so that all of its glomeruli might be stained and estimated according to Nelson. Thus, in these experiments, while both kidneys received the dye intravitally, the left was subjected to additional treatment with it in order to stain those glomeruli not reached by intravital injection. The numbers of glomeruli reached by the dye in the right kidney were 65 and 76 per cent, respectively, of the total numbers in the left.

In eleven of this series of thirteen control experiments it is seen that the numbers of glomeruli open to the circulation range from 56 to 91 per cent. In this group are included one in which the renal vascular action of Janus green B was overcome, two in which India ink was substituted for Janus green B, and two in which the animals were not eviscerated. The range of values derived from frozen sections is not materially different from that derived from the Nelson method. The results at once testify to the adequacy of the method and are adapted for comparison with similar results in animals in which experimental alterations in the renal circulation have been induced.

TABLE II.

Estimations of Glomeruli Open to the Circulation during Changes in Renal Circulation.

Experiment No.	Sex.	Weight.	Change in blood pressure.	Change in urine flow.	Glomeruli stained.		Percentage of stained glomeruli in vitally stained kidney.		Experimental procedure.
					In control kidney.	In vitally stained kidney.	From counts.	From frozen sections.	
		kg.	*mm. Hg*	*drops per min.*					
29	F.	1.6	62–122	5–0	214,100 (3)	11,664 (6)	5	3.6	Adrenalin 1:10,000.
30	"	2.1	80–124	7–7	293,996 (3)	48,042 (3)	16	9.5	" 1:10,000.
32	"	1.3	70–96	2.5–2	221,383 (3)	80,908 (3)	36	31	" 1:100,000.
33	M.	2.5	86–96	7–7	175,240 (3)	142,839 (3)	81	76	" 1:250,000.
53	F.	1.6	92–102	1–1	200,606 (4)	62,643 (3)	31	27	" 1:100,000. Oncometer and renal blood flow.
56	"	1.5	94–140	1.5–0.5	219,378 (3)	68,354 (4)	31	38	Adrenalin 1:50,000. Oncometer and renal blood flow.
57	M.	1.7	89–130	14–0	196,043 (4)	169,454 (4)	86	96	Adrenalin 1:25,000. Oncometer and renal blood flow.
65	"	2.0	120–184	1–0.5	162,736 (3)	58,382 (4)	36	25	Adrenalin 1:10,000. Fall in kidney volume after Janus green B prevented.
41	F.	1.4	63–136	3–0	109,931 (2)	28,911 (3)	14	29	Adrenalin 1:10,000. India ink instead of Janus green B.
25	"	1.7	54–60		143,149 (3)	72,940 (4)	50	87	10 per cent carbon dioxide.
22	"	2.3	76–110		184,876 (3)	17,383 (5)	9	11	Carbon dioxide.
47	M.	1.2	100–112	1–0	191,087 (3)	120,526 (3)	63	79	Splanchnic stimulation.
48	"	1.4	62–74	0.6–0	174,841 (3)	86,563 (3)	49	46	Stronger splanchnic stimulation.
20	F.	1.7	88–50		216,716 (3)	210,616 (3)	97	100	Bled 18 cc. in 2 min. 20 sec.

TABLE II—*Concluded.*

Experiment No.	Sex.	Weight.	Change in blood pressure.	Change in urine flow.	Glomeruli stained.		Percentage of stained glomeruli in vitally stained kidney.		Experimental procedure.
					In control kidney.	In vitally stained kidney.	From counts.	From frozen sections.	
		kg.	*mm. Hg*	*drops per min.*					
42	F.	1.8	88–32–60	3–0	135,083 (3)	127,480 (3)	94	95	Bled 19 cc. in 1 min. 40 sec. Allowed 3 min. to recover.
44	"	1.1	92–36	1–0	252,140 (3)	163,338 (3)	64	80	Bled 15 cc. in 7 min.
45	"	2.2	98–28	15–0	161,358 (3)	46,935 (4)	28	27	" 33 " " 6 "
27	M.	1.4	80–90	0.2-46	179,613 (3)	166,499 (3)	92	89	Caffeine + 10 per cent NaCl.
28	"	1.7	76–100	1–54	172,302 (3)	173,349 (3)	100	100	Caffeine + 10 per cent NaCl.
55	F.	1.3	78–84	2–14	209,265 (3)	208,320 (3)	100	100	Caffeine + Locke's solution. Oncometer and renal blood flow.

Figures in brackets following the number of glomeruli indicate the number of pieces of cortex counted.

Experimental Alterations in the Renal Circulation (*Table II*).

The means used to influence the renal circulation have been such as are known to cause marked change in it either by action on the vessels themselves or on the general circulation. Caffeine and NaCl, or caffeine and Locke's solution were chosen as renal vasodilators: to induce renal vasoconstriction or lessening in renal blood flow, we used adrenalin in various dosage, CO_2 inhalation, stimulation of the splanchnic nerve, and hemorrhage.

1. Vasodilatation.—In two experiments 20 mg. of caffeine + 4 to 6 cc. of 10 per cent NaCl, and in a third, 20 mg. of caffeine and about 15 cc. of Locke's solution were injected intravenously. In each case, arterial blood pressure decreased slightly, subsequently rising to a little above its original level, and profuse diuresis occurred. The kidney volume increased greatly in Experiment 55, the only one

of the three in which an oncometer was applied. Estimates of the number of glomeruli reached by injected Janus green B were 92, 100, and 100 per cent respectively. Practically identical figures were obtained in total counts of 500 glomeruli in frozen sections of the intravitally stained kidney (89, 100, and 100 per cent respectively).

2. Vasoconstriction.—In nine experiments, adrenalin in concentrations varying from 1:250,000 to 1:10,000 was injected into the ear vein at the approximately constant rate of 0.005 cc. per second. After the blood pressure had remained constant at its increased level for several minutes, the adrenalin injection continuing, the Janus green B injection was made. Stained glomeruli in the kidneys of these nine experiments numbered from 5 to 86 per cent of the total; in seven experiments the number was 36 per cent or lower. One experiment was made in which India ink was substituted for Janus green B, the adrenalin dosage chosen being such as to permit the expectation that approximately 5 per cent of the glomeruli would be stained; the actual estimation showed 13 per cent stained. Comparison of these figures with those in the control series (Table I) shows that in seven out of nine experiments, the number of open glomeruli was less than the lowest control figure.

In Experiment 25, the animal was made to breathe 10 per cent CO_2. Blood pressure rose from 54 to 60 mm. The glomeruli stained numbered 50 per cent of the total. In another experiment (22) a higher concentration (unknown), which caused much more marked rise of blood pressure, reduced the number of glomeruli stained to 9 per cent of the total.

Two experiments in which the left splanchnic nerve was stimulated, with resulting slight rise of blood pressure, showed staining of 63 and 49 per cent of the total number respectively.

Four experiments were made to test the effect of hemorrhage. In three of these the percentages of glomeruli stained were high (64, 94, and 97 per cent); in only one was the percentage reduced below the figures of the control series (28 per cent).

Relation of Renal Blood Flow to Number of Open Glomeruli (Table III).

In eight of the experiments already summarized, estimations were made of total blood flow through the kidney by Livingston's method (6). Dr. Livingston himself conducted the work of applying it in these animals, and we are deeply indebted to him for his collaboration. The method involves the periodic accurate measurement of the extent to which the kidney swells during occlusion of the renal vein for exactly $\frac{1}{2}$ or exactly 1 second. Four of these experiments were in the control group, one in the caffeine, and three in the adrenalin series. The results are collected in Table III.

The series is too short and the experiments in it are not sufficiently

representative to add as much as we had hoped to the information derived without the aid of blood flow estimations. It happens that the control experiment in which fewest glomeruli were stained, the two controls in which the greatest number of glomeruli were stained, and the adrenalin experiment in which the greatest number of glomeruli were stained are included in this group: hence, four out of the eight experiments may be regarded as exceptional in their respective groups from the standpoint of glomerular count.

TABLE III.

Estimations of Renal Blood Flow and of Glomerular Blood Flow.

Experiment No.	Weight of left kidney.	Blood pressure.	Change in kidney volume before Janus green B.	Urine flow.	Blood flow.			No. of open glomeruli.	Percentage of open glomeruli.	Remarks.
					Per min.	Per gm. per min.	Per open glomerulus per min.			
	gm.	*mm. Hg*	*cc.*	*drops per min.*	*cc.*	*cc.*	$\times 10^{-4}$ *cc.*			
50	8.5	84	0	6	32.0	3.7	1.88	170,833	77	Control.
51	6.0	78	+0.1	3	20.0	3.3	1.04	191,485	100	"
52	7.0	70	0	1	24.7	3.3	1.20	205,248	91	"
55	6.2	78		2	11.1	1.8				Before diuresis.
		84	+0.65	14	16.8	2.7	0.81	208,320	100	During "
57	7.5	89	−0.1	14	22.6	3.0				Before adrenalin.
		130	−2.0	0	7.8	1.0	0.46	169,454	86	During "
53	7.0	92	−0.05	1	13.4	1.9				Before adrenalin.
		102	−0.22	1	10.6	1.5	1.72	62,634	31	During "
54	6.5	82	−0.1	0.6	7.7	1.2	1.00	77,733	42	Control.
56	6.2	94	+0.22	1.5	13.5	2.2				Before adrenalin.
		140	−1.52	0.5	4.0	0.6	0.58	68,354	31	During "

Despite this fact, when six out of the eight experiments are considered, a rough parallelism is seen between percentage glomerular estimate and total blood flow. It is scarcely to be doubted that if blood flow figures were available in the other experiments this parallel-

ism would be much more striking. No parallelism exists between percentage glomerular estimate and average rate of blood flow per individual open glomerulus. The trends of the two curves indicate rather that the fewer glomeruli open, the greater is the blood flow through each. In the absence of sufficient data upon which a generalization can be based, we may point out the fact that individual experiments show that individual glomerular blood flow may be conspicuously high in kidneys in which less than one-third of the glomeruli are receiving blood.

SUMMARY AND DISCUSSION.

The results of thirteen control experiments, designed to show the number of glomeruli in the rabbit's kidney open to the circulation under the chosen experimental conditions without intentional interference, indicate the "normal" range to be from 42 to 100 per cent. Since ten of the thirteen results fall within the figures 56 and 89 per cent, we may take these figures as the chief basis for our discussion.

Three experiments only were made in which renal vasodilatation was produced by caffeine and salt. The percentage of open glomeruli found was in every case higher than any control except one. The results show without ambiguity that in rabbits, as in frogs, renal vasodilatation by caffeine is accompanied by increase in number of patent glomeruli. Our prime interest lay in the general question rather than in the action of individual substances in the group of vasodilators; hence this series was not extended further.

Among the experiments designed to test the effects of renal vasoconstriction are to be found nine in which adrenalin was injected, two in which CO_2 was inhaled, two in which the splanchnic nerve was stimulated, and four in which hemorrhage was induced. Not all are of equal value for our present purpose, inasmuch as the degree of certainty with which we can assume that renal vasoconstriction was actually produced is not the same in all.

Enough experience with the action of adrenalin on the kidney of the anesthetized rabbit is available to permit the assertion that the dosages used in the nine adrenalin experiments were sufficient to insure constriction of renal vessels.

Similar certainty exists in the experiment in which high concentration of CO_2 was used; less in the case of 10 per cent CO_2.

Stimulation of the splanchnic nerve in rabbits so frequently fails to produce results typical of direct constriction of renal vessels that we may regard the production of this effect in the two experiments in which this was done as doubtful. In the perfusion experiments by Richards and Plant (7) the reactions of the renal vessels to stimulation of the splanchnic nerve resembled those to intravenously injected adrenalin rather than to the direct excitation of constrictor fibers. In six rabbits in which Livingston subjected the nerve to varying degrees of electrical stimulation, in one only was distinct constriction of the renal vessels produced, and in this a latent period of 45 seconds occurred between the beginning of stimulation and the production of effect.[8] In one of our experiments a rabbit was used in which the superior cervical sympathetic ganglion on the left side had been extirpated months before. During the stimulation of the splanchnic nerve the left pupil was observed to dilate, showing increased adrenalin secretion. Hence, we are inclined to regard the two attempts to produce vasoconstriction by this means as having been largely unsuccessful.

In one experiment only of the four in which hemorrhage was induced was there unmistakable evidence of compensatory vasoconstriction which may have involved the renal vessels. The blood pressure curve of this animal showed rhythmically occurring waves of the Traube-Hering type and dyspnea was noted. In this the estimate of open glomeruli was 28 per cent.[9] In the other three experiments no such change occurred and no dyspnea was seen. In pithed frogs, hemorrhage alone of moderate extent is less apt to lessen the number of patent glomeruli than are any of the other constrictor agencies tried by Richards and Schmidt.[10]

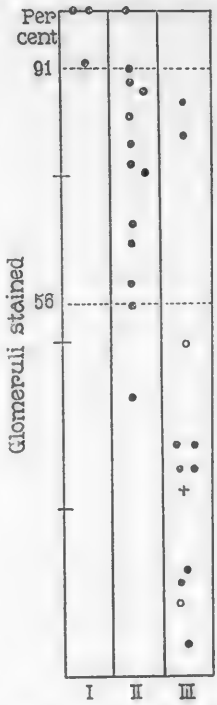
In this discussion, therefore, we lay little weight on the results obtained in the two experiments in which the splanchnic nerve was stimulated and on those of the first three hemorrhage experiments.

[8] Personal communication.

[9] In this experiment the distribution of stained glomeruli throughout the cortex was unusual and suggestive. In the extreme outer third almost no glomeruli were stained, in the extreme inner third almost all were stained, in the intermediate zone a few were stained. It is to be noted that in addition to vasoconstriction which presumably involved the kidney, arterial blood pressure in this experiment fell to 28 mm.

[10] Observation by Dr. Richards.

The chief conclusion to be drawn from these experiments is that which was anticipated from the observations of Richards and Schmidt on frogs, and of Khanolkar on rabbits; *viz.*, that in the rabbit, renal vasodilatation and renal vasoconstriction are usually associated with increase and decrease respectively in number of glomeruli through which blood flows (see Text-fig. 1). Analogous changes apparently occur in the capillary pathway in individual glomeruli. Hence renal function in mammals may be altered by changes in the extent of glomerular filtration surface to which the blood has access. Other conditions remaining the same, it is obvious that changes in extent of filtration surface must result in proportionate changes in urinary output. The figures for rate of urine elimination at the time of injection of the dye in these experiments are in substantial agreement with these statements (see Text-fig. 2).



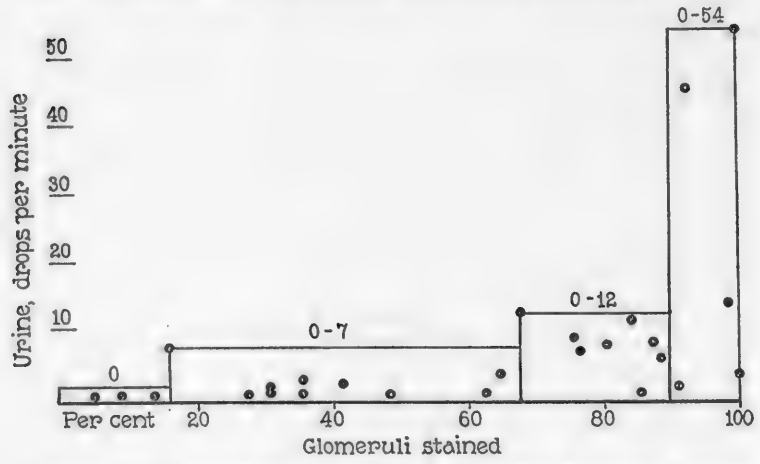
TEXT-FIG. 1. Percentages of open glomeruli in the kidneys in 28 experiments. I, renal vasodilatation; II, control; III, renal vasoconstriction. In Column III, black dots represent adrenalin experiments, circles CO_2 experiments, and the + a hemorrhage experiment.

Exceptions to our chief conclusion as stated have been encountered. In Experiment 57, 86 per cent of the glomeruli were open in a constricted kidney which was excreting no urine: in Experiment 30, 16 per cent were open in the kidney which was eliminating seven drops per minute: the outputs of the kidneys in the caffeine experiments were far higher than those of control kidneys in which comparable numbers of glomeruli were open.

In considering these exceptions, account must be taken of the fact that other conditions do not commonly remain constant. When a renal vasodilator is introduced we conceive not only of possible increase in extent of accessible glomerular surface, but also of increase in glomerular pressure and increased rate of renewal of fluid in contact with glomerular membranes.

Hence the response is greater than can be accounted for by any one factor alone.

A basis of experiment exists in support of the belief that usually sufficient differences in physiological state exist among the small arteries and arterioles of the kidney so that a constrictor influence, exerted equally upon all, elicits various degrees of response (1). Closure of some, continuing patency of others, results. Blood flow and blood pressure in the glomeruli which are supplied by the vessels which remain open may be decreased, increased, or unchanged accord-



TEXT-FIG. 2. Relation of urine flow to percentage of open glomeruli in twenty-six experiments.

ing to the relation between the degree of reaction in those vessels and the height of arterial blood pressure. It is not to be expected that urinary outputs will uniformly vary with the number of glomeruli remaining open. Rapid blood flow and high glomerular pressure in relatively few glomeruli may result in more urine than slow flow and low pressure in many. This argument is implicit in Hermann's original statement and is completely in harmony with the result of direct observation in the frog. It is supported by the data of Experiment 30.

In Experiment 57, however, urine was suppressed by adrenalin

when 86 per cent of the glomeruli remained open. The kidney in this experiment was highly diuretic before the adrenalin injection. We may, therefore, assume that all or nearly all of the glomeruli were open and that intermittent contractions of the arterioles were minimal; hence, that the physiological state of the vessels concerned was more nearly uniform than is conceived to be the case when only a fraction of the glomeruli are open and in which intermittent contractions and relaxations must be pronounced. Thus a relatively uniform constriction was produced in all of such degree as to lessen materially glomerular pressure and blood flow, but insufficient to actually close more than a few afferent arterioles. In Experiment 33, the dosage of adrenalin was such as to permit the possibility that constrictor action may have been largely confined to efferent vessels (8).

While the exceptional results have been discussed at greater length than has been devoted to the majority of experiments, we believe that they do not constitute adequate ground for criticism of the chief conclusion as stated.

CONCLUSIONS.

1. Intravital staining of the glomeruli of the kidney can be accomplished by Janus green B provided the solution of this dye be injected into the aorta.

2. In a normal rabbit the two kidneys usually contain approximately the same number of glomeruli.

3. On the basis of these two observations, Nelson's method of estimating the number of glomeruli in a kidney has been adapted for showing the number of glomeruli which are open to the circulation at the moment of injection of the dye.

4. This adaptation has been used to determine the number of open glomeruli in the rabbit's kidney under various conditions. Wide variations in the number occur spontaneously and may be produced experimentally. Hence the extent of surface of glomerular membranes to which blood has access is variable.

5. Caffeine and salt solution, used as typical renal vasodilators, cause the number of open glomeruli to increase. All or nearly all of the glomeruli are open during the action of these substances.

6. Vasoconstricting agents, such as adrenalin and CO_2 inhalations,

usually reduce the number of glomeruli open to the circulation. If the vasoconstriction is pronounced the number of open glomeruli may be only 10 per cent, or less, of the total number in the kidney.

7. As a rule the number of open glomeruli, kidney volume, kidney blood flow, and urine elimination vary in the same direction; but there are exceptions to this generalization.

8. A decrease in kidney volume may be accompanied by one of the following vascular phenomena: (*a*) the number of open glomeruli may be reduced but the average blood flow per glomerulus continue high; (*b*) the number of open glomeruli may remain high, the constriction simply diminishing blood flow through them; (*c*) a reduction in number of open glomeruli may be accompanied by a reduced blood flow through those still remaining open.

We are indebted to Dr. A. N. Richards for the suggestion which led to this work, and for his collaboration in the preparation of this paper.

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EXPLANATION OF PLATES.

PLATE 29.

FIG. 1. Experiment 55. Injection of Janus green B during the action of caffeine Upper curve = kidney volume. Calibration of bellows is indicated. Second curve = blood pressure. At A, 0.75 cc. of saturated Janus green B was injected into the aorta. At B the aorta was clamped. The corresponding points are marked on the kidney volume curve. In this experiment all the glomeruli were stained.

FIG. 2. Experiment 53. Kidney volume, blood pressure, and urine. Between the two parts of the figure injection of 1:100,000 adrenalin was begun and continued to the end of the injection of Janus green B at A. At B the aorta was clamped. 31 per cent of the glomeruli of this kidney were stained.

FIG. 3. Experiment 65. Kidney volume, blood pressure, and urine. Between the two parts of the figure injection of 1:10,000 adrenalin was begun and continued to the end of the injection of Janus green B at A. 36 per cent of the glomeruli of this kidney were stained. The solution of the dye contained 20 mg. of caffeine, which prevented further vasoconstriction by the dye.

PLATE 30.

Photographs of crushed pieces of cortex of vitally stained kidneys.

FIG. 4. Staining of glomeruli and arteries from completely stained kidney. × 44.

FIG. 5. Experiment 55 (caffeine); 100 per cent stained. × 52.

FIG. 6. Experiment 54 (control); 42 per cent stained. × 52.

FIG. 7. Experiment 44 (hemorrhage); 64 per cent stained. Distribution of stained glomeruli in this preparation resembles that to which attention is drawn in the foot-note to page 654. About × 21.

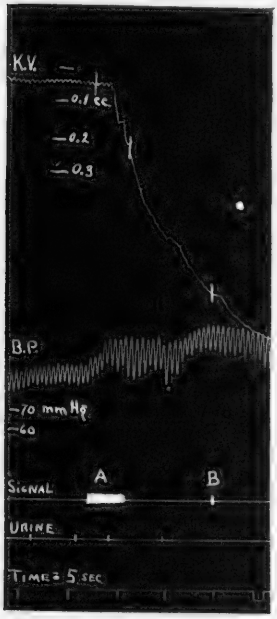
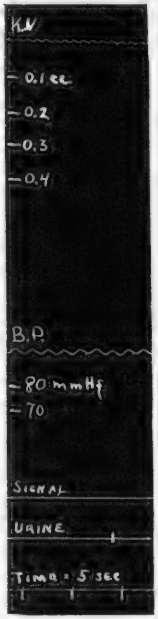


FIG. 1.



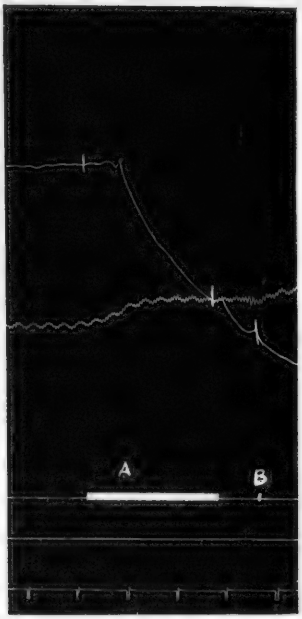


FIG. 2.

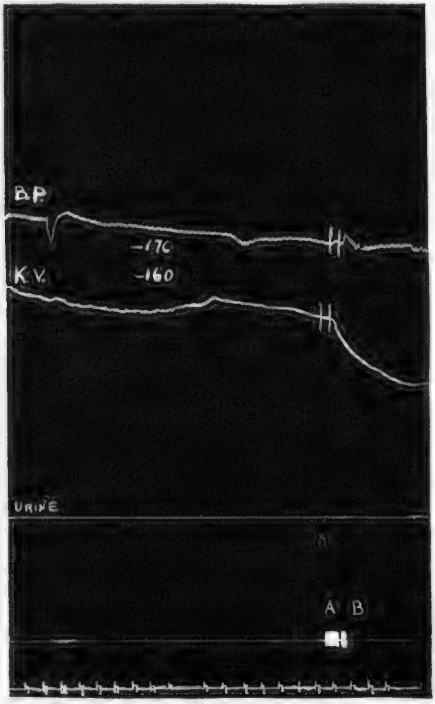


FIG. 3.

(Hayman and Starr: Distribution of blood in mammalian kidney.)

FIG. 4.

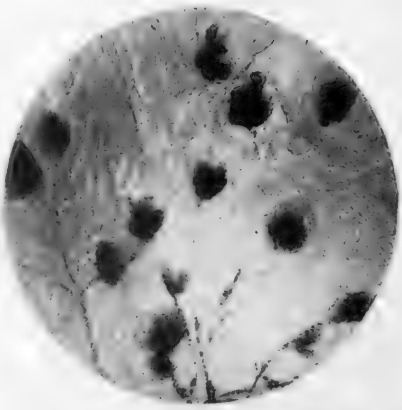


FIG. 5.

FIG. 6.

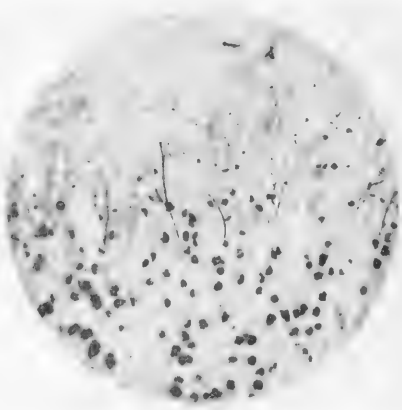


FIG. 7.

(Hayman and Starr: Distribution of blood in mammalian kidney.)

THE PHYSIOLOGICAL RESPONSE OF THE CIRCULATORY SYSTEM TO EXPERIMENTAL ALTERATIONS.

I. The Effect of Intracardiac Fistulæ.

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Plates 31 and 32.

(Received for publication, July 13, 1925.)

The cause of cardiac hypertrophy and dilatation continues to be a subject of opposing hypotheses and divergent opinions. Careful study of the heart and circulation in so called cardiovascular renal disease has failed to solve the puzzle of its many conflicting manifestations. Arteriosclerosis—increased peripheral resistance—cardiac hypertrophy is an alleged sequence of events, the many exceptions to which make an exact interrelationship difficult to determine. "Idiopathic hypertrophy of the heart" and "essential hypertension" represent additional familiar entities that remain unexplained.

In studies on arteriovenous fistulæ, both peripheral and intracardiac, evidence has been obtained indicating that an increased volume flow through the chambers of the heart is an effective stimulus to dilatation and hypertrophy, in spite of the decreased peripheral resistance incident to the fistula. Following the establishment of a large peripheral arteriovenous communication, as between the femoral or iliac vessels, the blood flow through the heart is practically doubled (1). Clinically and experimentally, dilatation and hypertrophy invariably follow such a fistula if it is sufficiently large (2) and of sufficiently long duration (3).

A long standing patent ductus arteriosus, unaccompanied by other complications, affects the heart in one of two ways, depending upon the direction of the blood flow through the abnormal communication (4). If the blood passes through the open ductus from pulmonary artery to aorta, a greater stream flows through the right

heart than through the left heart, and when the opening is sufficiently large, a dilatation and hypertrophy of the former are observed. On the other hand, if the blood deflected through the ductus flows from aorta to pulmonary artery, the greater stream passes through the left heart only, and the resulting dilatation and hypertrophy are limited to the left chambers.

Similar clinical observations have been made in the presence of other cardiac anomalies, as patent interventricular septum and patent foramen ovale, which frequently are associated also with pulmonary and aortic stenosis (5). The effect of these abnormalities is to direct the major stream of circulating blood through certain chambers and their corresponding vessels, the rest of the heart receiving a smaller volume of blood. Commensurate with this relatively increased blood flow, there occurs a dilatation and hypertrophy limited to these chambers, and a retarded development in that part of the heart receiving the lesser stream.

These clinical and experimental observations were sufficiently uniform and invariable to justify the presentation of the following hypothesis. The heart and blood vessels place themselves in harmony with an increased volume flow of blood through them, the former by dilatation and by hypertrophy, and the latter by dilatation.

Following an analysis of the circulatory effects produced by a congenitally patent septum, we undertook to establish such a lesion in the adult animal in order to observe the subsequent cardiac response, and to test the validity of the above hypothesis.

Method.

The experiments were performed under the strictest aseptic precautions. Some investigators in the physiology of the circulation have disregarded the obvious fact that "sacrifice" experiments cannot be accepted as telling the whole story in any study of physiological pathology, nor can the results of such experiments be applied to any pathological condition which requires the elapse of time for the development of the compensatory adaptation to its presence. This is particularly true in the complex and flexible circulatory system, which adapts itself in such an extraordinary manner to altered conditions within it. It is, therefore, only the survival experiment that can tell the true sequence of events, and such experiments require, in many instances, the technical equipment and operative skill of the trained surgeon.

The experimental methods used were those developed by Cutler and his co-

workers, Levine and Beck, in their studies on mitral stenosis and mitral insufficiency (6). The animals were anesthetized with ether by the intratracheal insufflation method with the Erlanger apparatus. The heart was exposed by an incision parallel to the left fifth rib, with or without resection of the rib. The pericardium was opened by a longitudinal incision parallel to the phrenic nerve. An apex stitch was applied for aid in manipulating the heart, and a knife valvulotome was introduced into the left ventricle through an avascular area at the apex. The interventricular septum was pierced preferably in its midportion, and an incision made toward the apex of the right ventricle in the longitudinal axis of the heart.

There were many technical difficulties. Several deaths occurred at the beginning, probably due to injury of the bundle of His. Some hearts were precipitated into "circus motion" with abrupt termination of contraction. One death was attributed to the complete division of an aortic leaflet, with the immediate development of aortic regurgitation in addition to the interventricular leak. Another death was due to mitral insufficiency following division of the chordæ tendineæ of the anterior mitral leaflet. Other deaths followed the establishment of septal defects that were excessively large.

RESULTS.

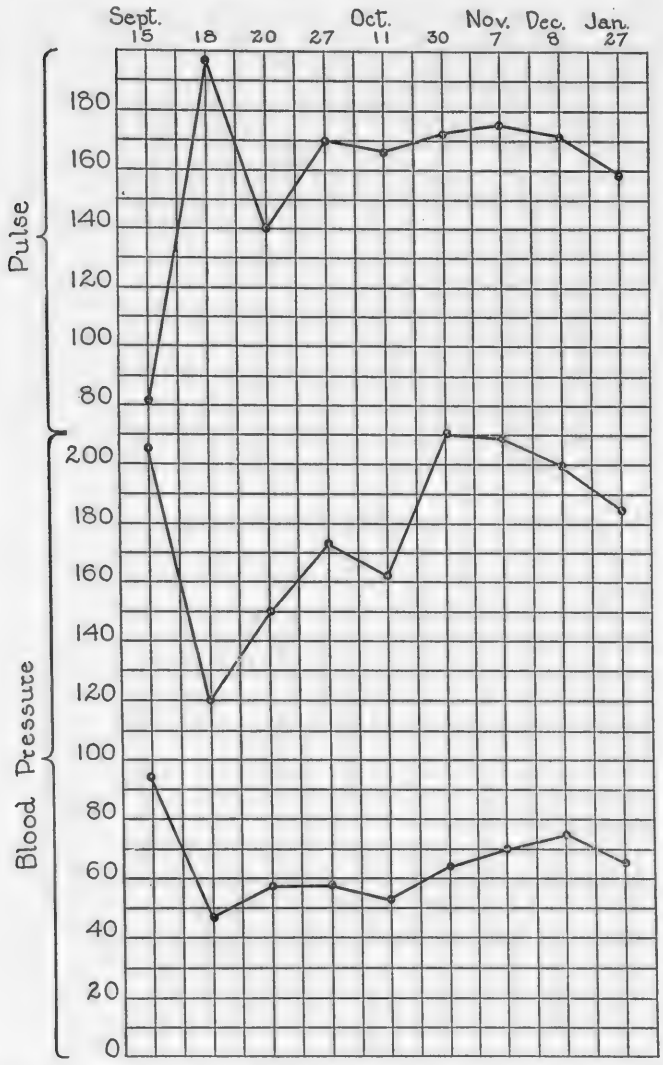
The following protocols present the important observations that were made.

Protocol of Dog X 11.—Weight 14 kilos. On September 16, 1924, an opening[1] of considerable size was established between the ventricles, accompanied immediately by an intense systolic thrill palpated over the right ventricle. (In none of the intracardiac fistulæ was the thrill felt over the left ventricle. This is interpreted as indicating that the flow of blood occurred from the left to the right side of the heart.) 2 hours after the operation the pulse rate was 202. A marked systolic thrill was felt over the right side of the chest, accompanied by a loud systolic bruit.

The following day the dog lay quietly in his cage, obviously ill; respirations 96, pulse 186; no cyanosis. The postoperative behavior of the pulse and the blood pressure is recorded in the accompanying chart (Text-fig. 1).

December 8, the pulse rate was 176. On auscultation, it appeared to several observers that the pitch during inspiration was higher than during expiration, and that the rate was slightly retarded during inspiration. The explanation for this phenomenon is not apparent. It is probable that the increased filling of the heart from the venæ cavæ during the inspiratory phase, with its accompanying negative thoracic pressure, may diminish the amount of blood flowing

[1] In this and the following experiments all operations were performed under ether anesthesia.



TEXT-FIG. 1. (Dog X 11.) Behavior of blood pressure and pulse following the establishment of a septum defect on September 16. Note fall in blood pressure and great acceleration in pulse rate persisting for several days with subsequent gradual recovery in blood pressure but permanent elevation in pulse rate.

through the defect and thus account for these changes in rate and pitch, an assumption dependent upon the observation that the bruit accompanying arteriovenous fistulæ is lower pitched in tone and the pulse rate more rapid when the flow through the fistula is large than when it is small. At no time were we able to recognize a second sound, either pulmonic or aortic, both being obscured by the systolic bruit.

On January 27 the animal was exercised. After a run of about 400 yards, it vomited and went into collapse. Immediately after the exercise the pulse was 220, and respirations 100.

Blood Volume Determinations.—The volume of the circulating blood was determined by the intravenous injection of brilliant vital red (7). Unfortunately, no dye was available for blood volume determinations before the operation for the establishment of the interventricular defect. The normal volume of a dog weighing 14 kilos, according to the dye method, is approximately 1400 cc., as the following figures indicate.

Weight of animal.	Blood volume.
kilos	*cc.*
11.1	1255
13.1	1357
13.6	1439
17.7	1867
20.0	2119
22.0	2166

On December 23, 1924, this dog had a blood volume of 2103 cc. A second determination made January 6 was 2096 cc. Compared with that of a normal dog ot the same weight, the blood volume of this animal had increased 700 cc. Allowing 200 cc. as a margin of error which the method may entail, it is seen that the establishment of an interventricular communication must have been accompanied by a definite increase in the volume of the circulating blood.

Roentgenographic studies exhibited a gradual increase in the size of the heart (Fig. 1). Before operation the heart had a transverse diameter of 7.3 cm. October 14 this had increased to 8.3 cm., December 23 to 8.9 cm., and February 9 to 9.6 cm.

February 10 an attempt was made by operation to reverse the flow through the opening by applying an aluminum band to the pulmonary artery. When the heart was exposed at the operation it appeared enormous, the enlargement being due mostly to the increased size of both ventricles. However, it was manifest that the left auricle entered into this enlargement also—the ear was prominent and appeared at least four to five times the normal size. It covered the anterior aspect of the upper third of the left ventricle. In contrast to this was the very small right auricle, about one-half as large as the left auricle, whereas

normally it appears several times larger. The right auricle was almost completely obscured by the enlarged right ventricle and greatly dilated conus arteriosus. The attempt to reverse the flow through the septum defect was unsuccessful and the dog died while under the anesthetic.

At necropsy the heart was conspicuously large (Figs. 4 and 5). The ventricles were capacious, the walls were thicker than normal, particularly those of the right ventricle and the septum. The left auricle was large and prominent, as previously observed in the living state (Fig. 4). The opening between the two ventricles admitted a 30 mm. bougie. The aorta, where it left the heart, admitted a 24 mm. bougie, the pulmonary artery a 42 mm. bougie, indicating that these vessels had placed themselves in harmony with the volume flow of blood through them.

The right ventricular wall was definitely hypertrophied. It measured 6 mm. at its narrowest portion, and the right ventricular cavity measured 23 mm. in diameter. In a normal animal of the same weight and size, the right ventricular wall measured 4 mm. at its narrowest portion, and the cavity was only 8 mm. in diameter. The heart weighed 162 gm., whereas the heart of the normal animal weighed 118 gm. The lungs showed discolored areas of congestion. They were firmer than normal, and the trachea and bronchi contained white frothy fluid which was not blood-tinged. Microscopic sections of the lung revealed a marked and intense edema and congestion, with some fibrin deposits in the alveoli suggesting a long standing edema. There was only a moderate congestion of the liver and kidneys, without edema. The heart muscle showed large nuclei with square ends, corroborative microscopic evidence of a cardiac hypertrophy (8).

Protocol of Dog X 7.—Weight 18 kilos. On September 14, 1924, a septum defect was made in the usual manner. The heart rate was increased from 156 to 198, but the thrill and bruit immediately following the operation were only of moderate intensity. The pulse rate on the following day was 105, the thrill had disappeared, and only a slight systolic bruit was present. It was inferred from these observations that only a minor leak had been established. Accordingly, on September 29, on which date even the systolic bruit was no longer audible, the dog was reoperated upon and a second interventricular opening was made. The heart rate before operation was 100; after operation 210. A well marked thrill and bruit were present after the operation. The next morning the animal was found dead. At necropsy, 10 cc. of bloody fluid were found in each pleural cavity. The lungs everywhere were dark red in color. The bronchi contained considerable frothy fluid. The heart was dilated, and both ventricles were capacious. The opening between the ventricles admitted a 34 mm. bougie. The weight of the heart was 135 gm. Microscopic sections of the lung showed intense pulmonary edema and congestion. The liver and spleen showed only slight congestion.

Protocol of Dog X 10.—Weight 10.4 kilos. The usual technique for the establishment of an interventricular leak was employed. The pulse rate increased immediately from 120 to 220. The animal died during the night, within 12

hours after the operation. Thin bloody fluid had escaped from the nose. The lungs were markedly congested, with the consistency of liver. The interventricular opening admitted a 30 mm. bougie. Weight of heart 71 gm. Microscopic sections of the lungs showed very marked edema and congestion, whereas the edema and congestion of the liver and spleen were insignificant.

Protocol of Dog X 23.—Weight 12.2 kilos. On December 16, 1924, an opening between the ventricles was established, with an increase in pulse from 153 to 180, and the appearance of a faint thrill and an intense bruit. Following operation, the animal was very ill. 8 days later there was considerable purulent discharge from the nose and eyes, indicative of a severe attack of distemper. Death occurred on January 3. At necropsy, a little free purulent fluid was present in each pleural cavity. There were multiple circumscribed areas of discoloration and consolidation in both lungs about 1 cm. in diameter. The heart was not enlarged. On opening the heart the left ventricular cavity was normal. The opening between the ventricles, the entire right ventricular cavity, and the pulmonary artery were filled with a friable, yellowish vegetative mass, which was adherent to the sides of the ventricular opening, to the wall of the right ventricle, and to the pulmonary valves. Microscopically, this mass was made up of necrotic material, infiltrated with polymorphonuclear white cells and round cells. Sections stained for bacteria were negative. The presence of a purulent nasal discharge, due to distemper, with the subsequent development of endocarditis, is an interesting experimental duplication of the frequent clinical observation that death in many cases of congenital cardiac anomalies is due to a superimposed endocarditis. The lungs showed multiple areas of embolic consolidation. The absence of cardiac dilatation in the presence of a septum defect 26 mm. in diameter is dependent upon the obstruction to blood flow through the defect due to the vegetative mass filling the opening, the right ventricle, and the pulmonary artery.

Protocol of Dog X 25.—Weight 11.2 kilos. Preoperative pulse 76. On December 15, an interventricular defect was established, with an immediate increase in pulse from 136 to 156, and the appearance of a thrill over the right ventricle. The right and left ventricles appeared definitely larger at the end of the operation as compared with their appearance at the beginning. On January 8 a bruit and thrill of only moderate intensity were recorded. The pulse rate was 148.

Roentgenographic studies showed a moderate increase in the transverse diameter of the heart from 7.1 to 8.2 cm. On February 9 the animal was found dead following a fight with another dog. The heart was larger than normal (Figs. 4 and 5). The right ventricle was dilated, as was also the left auricle, which was abnormally large. The left ventricle was capacious. The opening between the ventricles was lined by glistening endothelium, and admitted a 26 mm. bougie. The aorta admitted a 34 mm. bougie, and the pulmonary artery a 40 mm. bougie. The heart weighed 152 gm. Microscopically, the lungs showed marked edema, with some congestion. The liver was not congested. This

animal survived the establishment of the defect only 7 weeks. The extent of the circulatory changes wrought by a defect depend not only upon its size, but also upon its duration.

Protocol of Dog X 26.—Weight 14.1 kilos. An opening was made between the ventricles, with the development of the usual thrill and bruit, and with an acceleration in pulse rate. The animal was found dead 18 hours later. Blood-tinged froth had escaped from both nostrils. There was no free fluid in the pleural cavity. The lungs were dark red throughout, and firm. The bronchi were filled with pink froth. The interventricular opening admitted a 32 mm. bougie. The heart weighed 122 gm. Microscopic sections revealed an intense edema and congestion of the lungs, and only moderate congestion of the liver and spleen.

Protocol of Dog X 27.—Weight 13.4 kilos. On January 8 an interventricular defect was established, with an immediate acceleration of pulse from 150 to 180, and the development of a thrill and bruit over the right ventricle. On January 9 the animal had a cough, but seemed in fair condition. The pulse rate was 180. It died 40 hours after operation. At necropsy a bloody mucous discharge from the mouth was noted. 3 ounces of blood-tinged fluid were present in each thoracic cavity. The left lung was firm and dark in color, with no air-containing areas. The right lung showed normal areas alternating with firm congested areas. The liver and spleen showed areas of congestion and increased consistency, alternating with normal areas. The right heart was dilated. The opening between the ventricles admitted a 32 mm. bougie. The heart weighed 119 gm.

Microscopic sections of the lung showed marked edema and engorgement of the blood vessels, with congestion. The liver and spleen showed congestion.

Protocol of Dog X 42.—Weight 14 kilos. Preoperative studies showed a pulse of 98, a blood pressure of 220/90. On March 4 an interventricular defect was established, with an immediate increase in pulse rate from 164 to 202. On the following day the blood pressure was 105/45, pulse 188. 2 days later the blood pressure had recovered to 130/70, pulse 168. A thrill and murmur were present over the right chest. Within 4 days the pulse rate dropped to 148, and by March 17 it had dropped to 92. On April 1 a blood pressure of 170/70, and a pulse of 90 were recorded. Upon this date the thrill and bruit had become very faint. These observations, together with the rapid recovery in blood pressure and pulse, indicated that only a small leak was present. On April 21 the bruit had become almost inaudible, and the thrill had entirely disappeared. On February 26, before operation, the blood volume was calculated as 1430 cc. On April 24, 7 weeks later, when practically all clinical evidences of a septal leak had disappeared, it was 1200 cc. On April 27 the animal was found dead, having been killed in fighting.

Both by roentgenographic and by direct examination, the heart had increased little in size. The right ventricle, however, appeared slightly larger than normal. The heart was opened and only a small scar indicated the location of the septum defect. It was covered by endothelium, but a 10 mm. bougie could be passed

after breaking through this delicate endothelial obstruction. The aorta admitted a 32 mm. bougie, the pulmonary artery a 36 mm. bougie.

Protocol of Dog X 2.—Weight 18.4 kilos. A large ventricular defect was established on September 8, 1924, accompanied by the immediate development of a marked thrill, an intense bruit, a lowered blood pressure, and a rapid pulse.

	Blood pressure.		Pulse.
	Systolic.	Diastolic.	
Sept. 8, 1924 (before operation)	180	90	72
Sept. 9, 1924	130	44	162
Sept. 11, 1924	166	56	162
Oct. 3, 1924	170	60	144

During the subsequent months, up to the time of the animal's death, the blood pressure readings remained at a systolic level of 160 to 180, and a diastolic level of 65 to 76. The pulse, however, remained abnormally rapid for several months only. On January 3 the rate was 148. There then occurred a gradual return to a normal rate of 80 to 110, as compared to a previous rate of 140 to 160. Repeated roentgenograms following the operation showed a gradual increase in the width of the heart shadow (Fig. 2). The transverse diameter of the heart before operation was 7.3 cm. September 29 this had increased to 8.3 cm.; October 30 to 8.7 cm.; November 29 to 9 cm.; January 8 to 9.4 cm.; April 1 to 10 cm. The activity of the heart was plainly visible over the chest wall, and the systolic thrill and bruit were always intense. As in X 11, this bruit was higher pitched during inspiration than during expiration, and the rate was slightly but definitely retarded during inspiration.

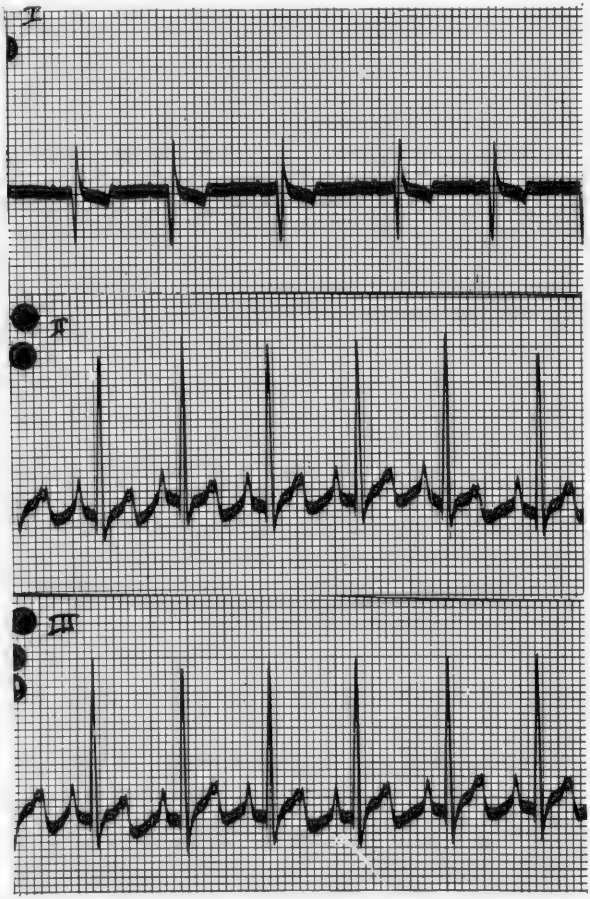
The blood volume determinations were as follows:

	cc.
Dec. 23, 1924	1635
Mar. 3, 1925	2075
Apr. 14, 1925	2100
May 8, 1925	2460

The increasing size of the heart was paralleled by a steadily increasing total blood mass. On April 27 an electrocardiogram revealed a relative right ventricular preponderance (Text-fig. 2).

The animal was killed on May 8. The necropsy revealed a greatly enlarged heart which weighed 224 gm. (Figs. 4 and 5). The heart of a normal large framed Airdale weighing 18 kilos appeared to be half the size of this heart, and weighed 162 gm. The left auricular ear was distended with blood and unusually prominent. The two ventricular cavities were capacious and dilated (Fig. 5). The pulmonary artery admitted a 40 mm. bougie, the aorta a 34 mm. bougie. The interventricular opening admitted a 28 mm. bougie, being slightly smaller

than the opening in X 11. The liver was enormous, firm, blue-bl[illegible] in appearance, and weighed 840 gm. The spleen was somewhat firm, not conspicuously congested, and weighed 48 gm. The liver of a normal dog of approximately the



TEXT-FIG. 2. Right ventricular preponderance observed in X 2, 8 months after establishment of septum defect.

same size weighed 390 gm., the spleen 36 gm. The lungs were firmer and darker in color than normal. Microscopic sections revealed an intense edema and congestion of the lungs, the alveoli and bronchi being filled with fluid. There

was also a marked congestion of the liver, spleen, and kidneys. The heart also exhibited microscopic evidences of hypertrophy, as indicated by the large nuclei with square ends. This evidence was more pronounced in the right ventricle than in the left ventricle.

Protocol of Dog X 34.—Weight 17.3 kilos. The preoperative blood pressure reading was 170/65, pulse 120. On January 31 an interventricular defect was established. 5 days later the blood pressure was 115/55, with a pulse of 176. March 3 slight recovery had occurred, the blood pressure being 150/70, the pulse 146, and on May 8 the blood pressure was 180/75, pulse 126, indicating a comparatively early return to normal readings. Roentgenographic studies revealed a well marked increase in the transverse diameter of the heart from 7.3 cm. on January 30, to 9.2 cm. on May 8 (Fig. 3).

The following blood volume studies were made.

	cc.
Jan. 28, 1925	1770
Mar. 3, 1925	1820
Apr. 14, 1925	1880
May 8, 1925	2140

An electrocardiogram on April 27 revealed a moderate relative right ventricular preponderance, which was not so striking as in X 2.

That the septum defect in this dog was smaller than in either X 2 or X 11 was evidenced by the rapid recovery of blood pressure, the early return of the accelerated pulse to a normal rate, the moderate enlargement of the heart, the lessened intensity of the thrill and bruit, and the moderate increase in blood volume.

The animal was killed on May 8. The heart was somewhat larger than normal (Figs. 4 and 5). It weighed 183 gm., whereas the heart of a normal similarly built animal weighed 162 gm. The ventricular cavities were moderately dilated; the pulmonary artery admitted a 38 mm. bougie, and the aorta a 34 mm. bougie. The left auricular ear was prominent. There were two small septum defects, one admitted a 16 mm. bougie, and the other a 10 mm. bougie. It is apparent that the combined cross-section size of these two openings was considerably smaller than the defect in either X 2 or X 11. The clinical manifestations in the latter two animals were much more pronounced than in X 34, and the effects upon the circulatory system observed at necropsy were less conspicuous in the presence of the smaller defect.

DISCUSSION.

From the foregoing protocols, it is apparent that the establishment of a defect in the interventricular septum resulted in certain invariable effects upon the circulatory system and particularly upon the heart, these effects depending entirely upon the size of the defect, and upon its duration. A defect larger than 30 mm. in circumference

in a heart 120 to 140 gm. in weight (X 7, X 10, X 26, X 27) was invariably fatal within 12 to 36 hours. The fatal issue was attended by the development of marked pulmonary edema and congestion. This congestion had the appearance of so called passive congestion, but it is evident that in these instances it followed not a *passive* but an *active* state of the circulation. The liver and spleen were frequently free of any congestion or edema.

The thrill and bruit which followed the establishment of the defect were directly proportional to the size of the defect, and, therefore, to the volume flow of blood through it. In the exposed heart they were limited to the right ventricle, and in the surviving animal they were most pronounced over the right side of the chest. They were limited to systole and completely obscured both the aortic and pulmonic second sounds.

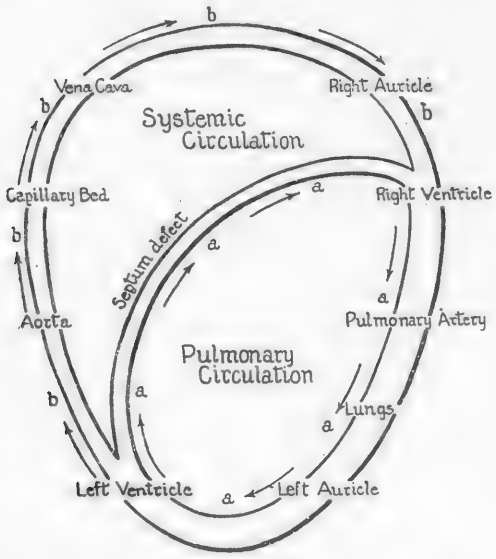
In the presence of the larger defects there was a slowly progressive dilatation of the heart, limited to the chambers and vessels through which the shortcircuited blood passed; namely, the left ventricle, right ventricle, the pulmonary artery, and the left auricle. The enlargement of the heart was demonstrable by roentgenograms and at necropsy. The right auricle and the aorta were unaltered or smaller than usual, conforming in size to the relatively decreased volume flow of blood through them. This was demonstrated most clearly in X 11.

There was a definite hypertrophy of the heart shown by an increase in the weight of the heart, and by an obvious thickening of the right ventricular wall (X 2, X 11, X 25, X 34). This hypertrophy is interpreted as being the physiological response to the increased effort necessary to propel forward an increased volume of blood. It cannot, in these instances, be due to an increase in peripheral resistance since this remains unaltered in relation to the right ventricle, and definitely decreased in relation to the left ventricle. Nor can it be due to pericardial adhesions, since numerous observations upon other experimental animals indicate that the adhesions, which invariably form following the opening and resuturing of the pericardium, have no effect upon the size of the heart.

Electrocardiographic studies on X 2 and X 34 revealed a relative

preponderance of the right ventricle, more pronounced in X 2, which possessed the larger defect.

In the presence of the large defects (X 2, X 11), a considerable increase in total blood volume was demonstrated. Dog X 34, with a moderate defect, showed only a moderate increase in blood volume. In Dog X 42, with a small defect which healed completely, no increase in blood volume was exhibited.



TEXT-FIG. 3. Scheme of circulation in presence of septum defect established experimentally in an adult animal.

The sequence of events subsequent to the establishment of a septum defect is interpreted as follows:

The presence of this direct communication between the arterial and venous systems provides two routes for the circulating blood (Text-fig. 3): (*a*) left ventricle, septum defect, right ventricle, lungs, left auricle; (*b*) left ventricle, aorta, capillary bed, right heart, lungs, left auricle. The difference in peripheral resistance between the pulmonic and systemic capillary beds, and the preponderance of the

left ventricle over the right, result in a division of the blood stream in the left ventricle, a part of the circulating blood being diverted through the defect during systole, the quantity depending upon the size of the opening. Commensurate with the volume of blood abstracted from the systemic circulation there occurs a marked fall in general blood pressure. If the amount of blood deflected is too large (Dogs X 7, X 10, X 26, X 27), the animal succumbs either from an excessive pulmonary congestion and edema, or from a lowered general blood pressure, or from a combination of both factors. If the animal survives the immediate effects of the defect, a gradual recovery in blood pressure is observed until the systolic pressure has again reached a normal level.

The factors to which may be attributed the reestablishment and maintenance of a blood pressure compatible with life are several. The pulse rate is immediately accelerated. That this great increase in pulse rate may gradually subside over a period of months was illustrated in our experimentally produced peripheral fistulæ, and also in the intracardiac fistulæ. In X 11, with a large defect, the pulse never returned to normal during the 6 months of observation. In X 2, with a slightly smaller defect, the pulse returned to normal after 5 months. In X 34, with a still smaller defect, the pulse returned to normal within 2 months, and in X 42, with a defect which closed spontaneously, the pulse returned to a normal rate within a month. This gradual return to a normal pulse rate is explained as follows:

The increased cardiac output necessary to compensate for the shortcircuited flow through the fistula is at first achieved by a more rapid rate of contraction of the heart. With the increased filling of the heart, provided by the diversion of the circulating blood into Circuit A, the heart is enabled to increase its output by this increased frequency of heart beat. This is the first, and the immediate compensatory adjustment for the abnormal leak. If the septum defect is large the volume of blood deflected into the shorter circuit is considerable. Whence its source? Immediately following the establishment of the fistula, it is obtained at the expense of the volume of blood flowing through the rest of the body; *i.e.*, through Circuit B. If the volume deflected is excessive the animal obviously cannot

survive, and a number of our experiments terminated fatally within 12 to 36 hours. The animals that survived were ill for a number of days, with a very low general blood pressure and a rapid pulse. A gradual recovery followed, due, according to our observations, to a second compensatory adjustment in the form of a gradual increase in total blood mass. The normal volume flow through Circuit B, temporarily decreased by diversion of a considerable part of the circulating blood into Circuit A, is slowly reestablished by increasing the total volume of blood. As this increase in blood volume approaches the point of full compensation for the shortcircuited flow, the pulse again returns to a normal rate.[2] The increased cardiac output necessary to maintain a normal general blood pressure is now attained by a slower rate of contraction, since the left ventricle, already dilated by a greatly increased filling, has developed a systolic discharge entirely sufficient to provide both for the septal leak and for the systemic circulation. Under these circumstances, complete equilibrium has been reestablished between Circuits A and B. The rapidity with which this equilibrium is reestablished depends primarily upon the size of the defect. The resistance offered to the flow of blood through the shorter circuit is the sum of that presented by the pulmonary bed and that provided by the septum defect itself. When any further increase in the volume flow through the shorter circuit is prevented by the limited size of the defect, resistance in the two

[2] The question of venous pressure in peripheral arteriovenous fistulæ is probably also dependent for a satisfactory explanation upon the basis of a *gradual adjustment*, much in the same way that the accelerated pulse rate returns to normal when complete equilibrium has been reestablished in the circulation. Immediately after the establishment of a peripheral fistula there is an increase in venous pressure proximal to the fistula, as numerous experiments have shown. As the vessels proximal to the fistula, including both the feeding artery and the *receiving vein*, undergo gradual dilatation to accommodate the increased flow through them, the increased quantity of blood delivered to the heart is maintained by an increase in the cross-section of the column of blood entering it. As a result, the increased venous pressure subsides without, however, any decrease in venous filling. Clinically, the cases of arteriovenous fistulæ in which the circulation has reached an equilibrium may show a normal *venous pressure*, as Lewis and Drury (9) contend, although actual *venous filling* per unit of time may be greatly augmented by an increase in the size of the column of blood entering the heart.

circuits is equalized. There will then be no further increase in the volume flow of blood through Circuit A, no further dilatation of that part of the circulatory system through which the deflected flow passes, and no further increase in total blood volume. It may then be said that complete compensation for the leak has occurred. In Dog X 11, in which a large defect was established, and in which the pulse never returned to a normal rate, it is assumed that complete compensation had not been attained when the animal was killed. The slightest effort in this animal produced complete collapse. It is likely that overdilatation of the heart through the mechanism of an increasing blood flow through Circuit A, and of an increasing total blood volume, might eventually have resulted in cardiac decompensation in this dog. May not decompensation be an inevitable development whenever dilatation outstrips hypertrophy?

In the presence of a peripheral or intracardiac fistula, the conditions in the heart conform exactly to the requirements which, according to Fick, are considered essential for the development of cardiac hypertrophy. Fick "holds that muscles elicit more or less work according to the degree to which they are stretched. He believes that hypertrophy can occur only if the stimulus to contraction, or the contraction itself, results in a stretching greater than normal" (10). The deflection of a considerable volume of blood into the shorter circuit provides for an increased filling of this part of the circulatory bed including the left auricle, the left and right ventricles. During each cardiac cycle, therefore, there occurs a greater stretching of the muscle fibers in these chambers than under normal conditions, and hypertrophy is an inevitable sequence to the large fistula, both theoretically and experimentally. It is apparent, however, from our experimental observations that hypertrophy of the heart is responsible only in small part for the cardiac enlargement that occurs. It is mainly a dilatation.

The mechanism that determines the various manifestations that follow in the wake of a fistula, whether peripheral or intracardiac, is the same in all cases, and is dependent upon an increased flow of blood through the shorter circuit. Our experiments indicate that the extent of these manifestations and the subsequent pathological changes depend upon the size of the fistula and upon its duration.

Additional evidence is presented by these experiments to substantiate the hypothesis that the heart and blood vessels place themselves in harmony with the volume flow of blood through them, the former by dilatation and by hypertrophy, and the latter by dilatation.

SUMMARY.

An abnormal communication, experimentally produced between the right and left ventricles, causes a deflection of part of the blood stream into the shorter pulmonary circuit. Proceeding *pari passu* with the increase in volume flow of blood through this shorter circuit, there occurs a gradual enlargement of the heart limited to that part of the circulatory system through which the deflected blood passes; namely, the left ventricle, the right ventricle, the pulmonary artery, and the left auricle. There is also a demonstrable hypertrophy of the right and left ventricles, which presumably is the result of the increased effort necessary to propel forward an increased volume flow of blood, since it cannot be attributed to an increased peripheral resistance.

Immediately after the production of the defect, the right auricle and aorta become smaller than usual, conforming in size to the decreased volume flow of blood through them. As full compensation for the deflected flow occurs by an increase in total blood volume, they return to their normal size. If full compensation has not occurred they remain smaller than normal (Dog X 11).

The changes incident to the establishment of an opening in the septum are entirely dependent upon the size of the defect, and hence, upon the extent of the volume of blood deflected into the shorter circuit. Commensurate with the volume of blood deflected, there is a fall in general blood pressure. If the animal survives the immediate fall in blood pressure, certain compensatory adjustments occur which reestablish a more normal blood pressure: (*a*) an immediate increase in pulse rate; (*b*) a gradual increase in total blood mass. The increase in blood volume is directly commensurate with the size of the defect. The pulse returns to a normal rate when complete compensation through an increase in blood volume has been attained.

It is suggested that the enlargement of the heart seen clinically in

so called "idiopathic hypertrophy," "essential hypertension," and also in certain cases of cardiorenal disease, may be due to an increase in total blood mass following some interference with the mechanism for its control. The seat of this impairment in blood volume control may be: (*a*) in a chemical alteration in the blood; (*b*) in a diseased function of the kidneys which may be responsible for a decreased elimination, or for a change in the chemical composition of the blood; or (*c*) in an abnormal stimulation of the organs producing the cellular elements of the blood.

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EXPLANATION OF PLATES.

PLATE 31.

FIG. 1. Increase in size of heart following establishment of septum defect on September 16: (a) September 15, 1924; (b) February 9, 1925.

FIG. 2. Great enlargement of heart in X 2 as revealed by roentgenogram. Defect established on September 8, 1924: (a) August 27, 1924; (b) March 9, 1925.

FIG. 3. Roentgenographic evidence of enlarged heart in X 34, following establishment of septum defect of 3 months duration: (a) January 30, 1925; (b) May 8, 1925.

PLATE 32.

FIG. 4. Hearts removed at necropsy illustrating marked enlargement in X 11 and X 2 with large septum defects, and less though definite enlargement in X 25 and X 34 with smaller defects of shorter duration. Note increase in size of ear of left auricle.

FIG. 5. The cardiac enlargement is due mainly to dilatation, and only partly to hypertrophy. Note thickness of right ventricular wall in X 11.

FIG. 1.

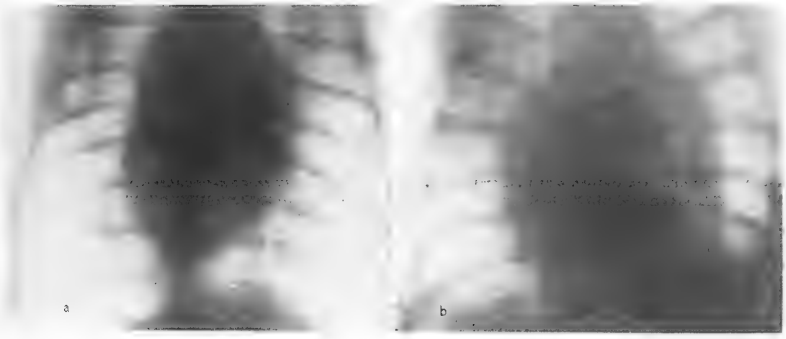

FIG. 2.

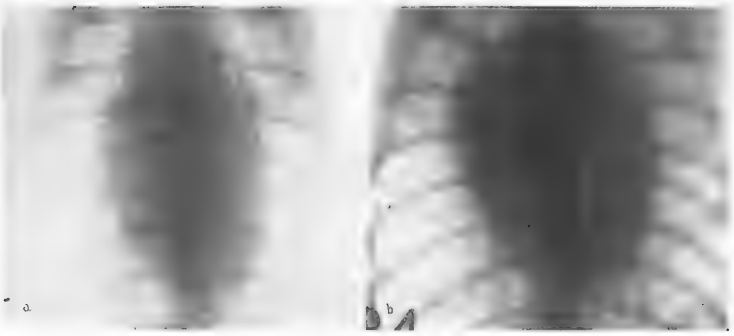

FIG. 3.

(Holman and Beck: Physiological response of circulatory system. I.)

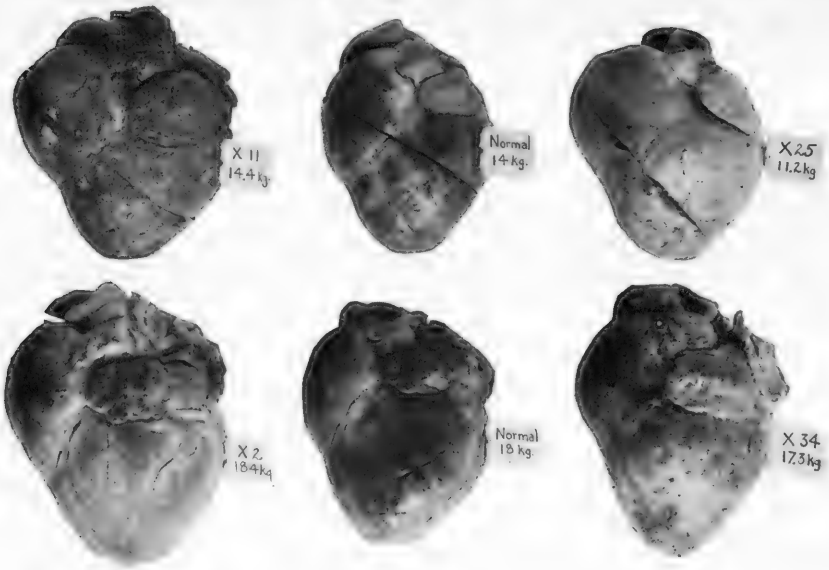

FIG. 4.

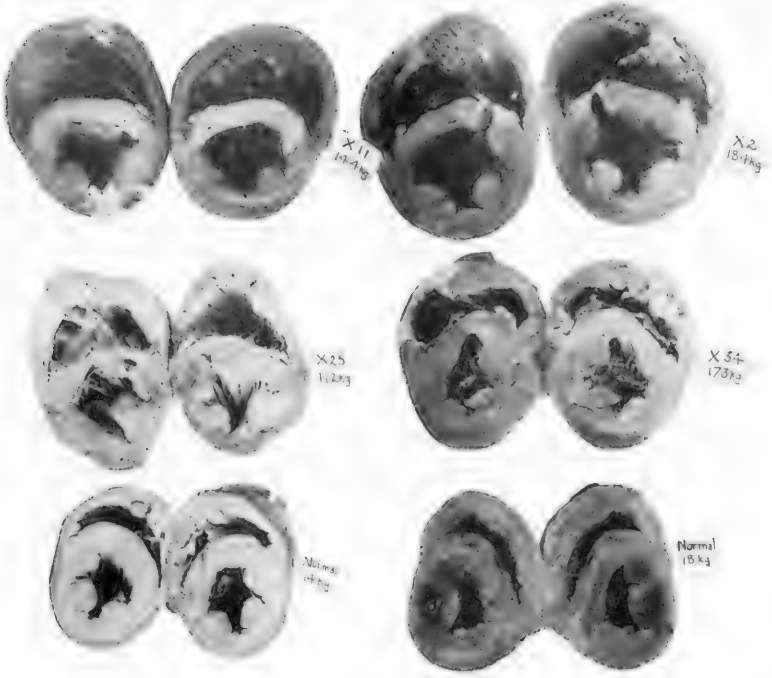

FIG. 5.

(Holman and Beck: Physiological response of circulatory system. I.)

THE PHYSIOLOGICAL RESPONSE OF THE CIRCULATORY SYSTEM TO EXPERIMENTAL ALTERATIONS.

II. The Effect of Variations in Total Blood Volume.

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Plates 33 and 34.

(Received for publication, July 13, 1925.)

"The blood volume should be regarded, not as aliquot part of the body weight, but as a physiological variable which is adjusted to the work required of it and to the size of the 'bed' which it occupies." Thus wrote Barcroft (1) in his interpretation of the intimate relationship of the spleen to the vascular system.

In our studies of peripheral fistulæ (2) and of intracardiac fistulæ (3), a definite increase in blood volume was demonstrated. This increase paralleled a gradually enlarging vascular bed as manifested by a dilating but efficiently working heart and by a dilatation of those vessels through which the blood, shortcircuited by the fistula, flowed.

Additional experiments were undertaken to determine the interdependence of the size of the heart and the total circulating blood volume, and to test further the validity of the hypothesis that "the heart and blood vessels place themselves in harmony with an increased volume flow of blood through them, the former by dilatation and by hypertrophy, and the latter by dilatation."

Method.

The experiments consisted of massive blood letting in one animal, and the transfusion of the whole blood so obtained into another animal. The experiments were performed under ether anesthesia without preliminary medication. The femoral vessels in each groin were exposed, one artery was connected with a mercurial manometer and the other was cannulated for bleeding. The right femoral vein was connected with an infusion bottle from which was introduced

either citrated blood or normal salt solution. Roentgenograms of the heart were taken with the tube fixed 5 feet above the dog and the plate at a fixed distance beneath. This permits accurate comparisons between successive roentgenograms. The exposure was sufficient to cover a complete cardiac cycle, including both systole and diastole. The results are presented in the following protocols.

RESULTS.

Experiment 1. Weight of dog 15 kilos.[1]

	Pulse.	Systolic blood pressure.	Transverse diameter of heart.
			cm.
Normal	192	152	8.4
300 cc. blood removed from right femoral artery	218	68	7.9
300 cc. more blood removed	228	50	6.7

The dog was then allowed to recover until the blood pressure was restored to a systolic level of 100 mm. Hg.

	Pulse.	Systolic blood pressure.	Transverse diameter of heart.
			cm.
400 cc. more blood removed	180	44	6.7
750 cc. salt solution infused in vein	198	96	8.0
250 cc. more salt solution infused	192	130	—
200 cc. blood removed	234	80	7.0
200 cc. blood removed	210	54	6.8
1300 cc. salt solution infused	204	116	8.6

The entire experiment lasted 2½ hours. The animal was saved and showed no ill effects from the loss of 1400 cc. of blood, an adequate circulating blood volume being evidently maintained by accessions from the tissue and organ fluids. The normal blood volume for a 15 kilo dog is about 1500 cc. of blood.

Experiment 2. Weight of dog 13 kilos.

The whole blood obtained from the preceding experiment was introduced into the left femoral vein, and the following observations were made (Fig. 1).

[1] In this and the following experiments all operations were performed under ether anesthesia.

	Pulse.	Systolic blood pressure.	Transverse diameter of heart.
			cm.
Normal	192	176	7.3 (Fig. 1 a)
450 cc. blood introduced	228	212	7.6 (Fig. 1 b)
450 cc. additional blood introduced	216	218	8.0
500 cc. additional blood introduced	214	196	8.1
800 cc. blood removed	180	106	6.9
300 cc. additional blood removed	164	70	6.7
300 cc. additional blood removed	192	54	6.1 (Fig. 1 c)
700 cc. blood reinfused	180	164	7.8
700 cc. additional blood reinfused	160	164	8.1
1100 cc. salt solution introduced	162	160	8.2 (Fig. 1 d)

The entire experiment lasted 2 hours. Adding 1400 cc. of whole blood to the circulation increased the diameter of the heart from 7.3 cm. to 8.1 cm. Removing the same volume of blood almost immediately resulted in a reduction in heart size from 8.1 cm. to 6.1 cm., indicating that the circulatory system had very rapidly disposed of part of the circulating blood volume to accommodate the blood added from without.

In the following experiment the animal was bled for 800 cc. of blood which was reinfused into the same animal 20 minutes later.

Experiment 3 (Fig. 2).

	Pulse.	Systolic blood pressure.	Transverse diameter of heart.
			cm.
Normal	204	134	6.3 (Fig. 2 a)
800 cc. blood removed from left femoral artery	174	28	4.9 (Fig. 2 b)
500 cc. blood reinfused 20 min. later	174	110	7.2 (Fig. 2 c)
300 cc. additional blood reinfused	144	132	7.2 (Fig. 2 d)
800 cc. blood removed	162	36	5.6
1200 cc. salt solution infused	156	90	7.7

Following the reinfusion of 800 cc. of blood into this animal it was noted that the swing of the lever recording the blood pressure had greatly decreased, suggesting a relatively greater increase in diastolic pressure than in systolic pressure with a diminution in pulse pressure. The exposed femoral arteries appeared to be dilated to twice their normal size and the roentgenograms gave evidence of a heart increased in diameter from 6.3 cm. to 7.2 cm. It is suggested that the diminished pulse pressure and the high diastolic pressure may be explained on the basis of an excessive filling of the entire vascular bed with blood.

In this instance, there occurred during bleeding a replacement of the blood volume by accessions from the tissues and probably also from such normal reservoirs of blood as the liver, spleen, and bone marrow By reinfusing the blood previously withdrawn there is a temporary increase in the blood mass contained in the vascular system as indicated by the dilatation of the heart and blood vessels.

It is also suggested that the diminished pulse pressure may be partly ascribed to the limiting action of the pericardium which prevents any great contractile excursion of a heart tensely dilated with blood.

Experiment 4. The whole blood obtained in Experiment 3 was infused into the femoral vein of a normal animal with the following results.

	Pulse.	Systolic blood pressure.	Transverse diameter of heart.
			cm.
Normal	156	96	7.8
500 cc. of whole blood infused	162	116	8.0
500 cc. more blood infused	162	124	8.6
1100 cc. salt solution infused	174	144	8.5
500 cc. blood removed	138	126	7.6
200 cc. additional blood removed	150	106	6.7

Immediately after completing the infusion of 1100 cc. of whole blood and 1100 cc. of salt solution, acute pulmonary edema developed, as shown by the loss of 200 to 300 cc. of frothy, bloody fluid from the mouth. The loss of this frothy fluid by mouth ceased immediately after the last bleeding.

DISCUSSION.

From the evidence obtained in these four experiments, the following conclusions are justified.

1. That the size of the heart conforms quite accurately to the volume of circulating blood.

2. That the addition of whole blood to the circulation of a normal dog increases the size of the heart, elevates the general blood pressure, and accelerates the pulse.

3. That massive blood letting reduces the size of the heart and lowers the general blood pressure to a degree commensurate with the volume of blood removed.

Interesting evidence was also obtained corroborating previous ob-

servations that the body has a rapidly adaptable mechanism for adjusting itself to an abrupt increase in blood volume. In Experiment 2, 1400 cc. of whole blood were added to the circulation with a rise in blood pressure and an increase in the size of the heart. Bleeding the animal almost immediately by an equal amount, 1400 cc., resulted in a marked reduction in the size of the heart and in blood pressure, indicating that the circulation had rapidly accommodated itself to the increased blood volume, not merely by a vasodilatation of the peripheral vessels (4), but by an actual egress of fluid from the circulatory system, presumably into the tissues or into the normal blood reservoirs, such as the spleen and the liver (5).

4. That the body has a remarkable blood volume reserve. In Experiment 1 the loss of 1400 cc. of blood had little effect upon the dog when the system was refilled with salt solution. In Experiment 3 the reinfusion of 800 cc. of whole blood, removed 20 minutes previously from the same dog, resulted in a marked dilatation of the entire vascular bed as indicated by an increase in the diameter of the heart from 6.3 cm. to 7.2 cm., and by a dilatation of the exposed vessels. As our other experiments have shown, a considerable volume of blood must be added to the circulation to produce such a dilatation. In Experiment 4 the addition of 500 cc. of blood increased the diameter of the heart only 2 mm., whereas 1000 cc. were required to increase the diameter 8 mm. The blood volume in Experiment 3 was increased temporarily following the reinfusion by an accession of blood and fluid from the tissues and from the normal blood reservoirs, accessions which were necessary during the bleeding to replace the blood withdrawn.

5. That massive plethora can produce an acute pulmonary edema with a transudation of blood through the pulmonary vessels (Experiment 4). This is probably the result of overdistention of the pulmonary vessels.

Although these are acute experiments, they manifest in many respects the same changes that characterize certain cardiovascular phenomena, as, for example, enlargement of the heart accompanied by hypertension. May not the phenomena observed in these experiments and in certain clinical entities have the same causative factor in common; *i.e.*, an increased total blood mass?

The Effect of Eliminating the Restrictive Action of the Pericardium.

In Experiments 3 and 4, it was noted that the acute dilatation of the entire vascular bed by plethora resulted in a diminished pulse pressure due, presumably, to a relatively greater increase in diastolic pressure than in systolic pressure. It was suggested that the dilated heart was limited in the excursion of its contraction by a restrictive action of the pericardium.

To determine what part the pericardium may play in limiting an acute dilatation of the heart and in restricting its contraction, the following experiments were undertaken.

Experiment 5 (Fig. 3).

700 cc. of whole blood were obtained from a normal animal. A second animal was anesthetized by intratracheal insufflation and the heart was exposed. The left femoral vessels were exposed and cannulated. The normal heart had a diameter of 7.2 cm. After the infusion of 1200 cc. of whole blood and salt solution, the diameter of the heart increased to 8.4 cm.

The pericardium was then opened by a longitudinal incision through which the heart bulged with almost explosive effort. It became markedly dilated and the left auricular ear became large and tense. Numerous dropped beats and extrasystoles marked the opening of the pericardium and the subsequent dilatation of the heart chambers. The excursion of the recording lever increased immediately from 7 mm. to 9 mm. and within 20 minutes to 15 mm. The heart was so large that an attempt to approximate the margins of the pericardium inclosed only one-half the heart. The roentgenogram showed a heart with a diameter of 9.1 cm. The dog was bled for 750 cc., and the diameter of the heart decreased to 6.4 cm. The pericardium was closed by interrupted silk sutures. The 750 cc. of blood were returned to the animal, resulting in an enlargement of the heart to 7.8 cm. (Fig. 3 a). At this point the amplitude of excursion of the recording lever had fallen from 7 mm. to 4 mm., accompanying the greatest filling of the vascular bed.

The pericardium was again opened, with the reappearance of numerous dropped beats and extrasystoles, and with an extraordinary dilatation of all the chambers of the heart. The transverse diameter of the heart increased to 8.7 cm. (Fig. 3 b).

The amplitude of the recording lever also increased immediately from 4 mm. to 7 mm.; within 3 minutes to 10 mm., and within 10 minutes to 12 mm., where it remained until the animal was killed.

Experiment 6.

The above experiment was repeated with the following observations. A heart

9.1 cm. in diameter was distended by the infusion of 800 cc. of whole blood to a transverse diameter of 9.6 cm., the pericardium being intact. Immediately after incising the pericardium the heart expanded to a width of 11.2 cm., with a tense dilatation of all its chambers.

CONCLUSIONS.

The pericardium presents a limiting or constricting action to acute dilatation of the heart. The diastolic pressure tends to approach the systolic pressure in a circulatory system distended with blood. The limiting action of the pericardium may be responsible for the small differences in the size of the heart noted by Meek and Eyster in their studies on the effect of plethora.

The views concerning the function of the pericardium are divergent. That it may restrict the heart in cases of acute dilatation is shown in the above experiments. If the tension upon the pericardium be exerted over a prolonged period of time, as occurs in cases of pericardial effusion, the pericardium readily enlarges. If, however, the intrapericardial pressure should at any time equal the pressure in the venæ cavæ, blood would no longer enter the heart and the condition would become fatal. In recovery experiments, pericardiectomy was followed by no demonstrable effect upon the general health of the dog, upon the response to exercise, or upon the size of the heart (6).

The Effect upon the Heart of a Redistribution of the Normal Blood Volume.

Previous experiments demonstrated remarkable changes in the size of the heart with variations in the total mass of the circulating blood. Equally significant effects upon the heart were produced by procedures which merely changed the *distribution* of the circulating blood without altering its volume. In general, the blood in the circulatory system is distributed between: (*a*) the heart and central vessels; and (*b*) the periphery, including the splanchnic area. Theoretically, an alteration in the peripheral system, either by a contraction or by a dilatation of its bed, should produce the reverse change in the heart. The following experiments were undertaken to test this point of view. Dilatation of the peripheral bed was effected by the inhalation of

amyl nitrite and by the injection of histamine. Contraction of the peripheral bed was accomplished by the injection of adrenalin or by an increase in intracranial pressure.

Experiment 7. The effect of amyl nitrite, adrenalin, and histamine upon the size of the heart (Fig. 4).

	Pulse.	Blood pressure.	Transverse diameter of heart.
			cm.
Normal	198	120	7.1 (Fig. 4 a)
Amyl nitrite (three ampules inhaled)	204	56	6.5 (Fig. 4 b)
Partial recovery from amyl nitrite	204	96	6.8
Complete recovery	192	120	7.0
15 minims adrenalin (1:1000) intravenously.			
25 sec. later	210	190	7.3
90 " "	180	176	7.0
130 " "	204	74	6.4
Recovery.			
20 min. later	180	116	6.2
12 mg. histamine intravenously	144	44	6.2
90 sec. later	150	50	5.9
10 minims adrenalin intravenously	140	108	6.7

Experiment 8. The effect of amyl nitrite and adrenalin upon the size of the heart.

	Pulse.	Blood pressure.	Transverse diameter of heart.
			cm.
Normal	145	120	8.0
Amyl nitrite by inhalation	205	96	7.7
Recovery	175	140	7.7
Amyl nitrite	225	120	7.2
Recovery	195	136	7.2
Adrenalin, 15 minims intravenously	150	280	8.2
3 min. later	180	96	7.3
Recovery	190	110	7.3

Experiment 9. The effect of histamine and adrenalin upon the size of the heart (Fig. 5).

	Pulse.	Blood pressure.	Transverse diameter of heart.
			cm.
Normal	156	110	6.5 (Fig. 5 a)
10 minims histamine intravenously.			
50 sec. later	168	46	6.0
150 " "	156	44	5.6 (Fig. 5 b)
30 min. "	150	100	6.1 (Fig. 5 c)
7 minims adrenalin intravenously.			
50 sec. later	198	178	7.1 (Fig. 5 d)
180 " "	192	100	6.2
40 min. "	174	110	6.3

Experiment 10. The effect of adrenalin upon the size of the heart.

	Pulse.	Blood pressure.	Transverse diameter of heart.
			cm.
Normal	186	122	6.3
10 minims adrenalin.			
60 sec. later	180	196	7.0
20 min. "	192	104	6.4

It is interesting to note a confirmation in these studies of an observation made by Gordon and Wells that after the first effect of the injection of adrenalin has subsided, there is a temporary fall in blood pressure below the level preceding the injection of adrenalin. Accompanying this fall there is a definite demonstrable reduction in heart size as our records show.

As the blood pressure again recovers, there is a recovery also in the size of the heart. The fall in pressure is explained on the basis of a dilatation of the peripheral bed following the very temporary constriction by adrenalin, evidence which contraindicates the use of adrenalin as a means of combating shock.

Experiment 11. The effect upon the heart of increased intracranial tension produced acutely by the injection of normal salt solution into the extradural spaces of the skull (Fig. 6).

	Pulse.	Blood pressure.	Transverse diameter of heart.
			cm.
Normal	168	112	6.6 (Fig. 6 a)
Moderate increase in intracranial pressure	156	120	7.3 (Fig. 6 b)
Great increase in intracranial pressure	120	156	7.7 (Fig. 6 c)

Experiment 12.

	Pulse.	Blood pressure.	Transverse diameter of heart.
			cm.
Normal	180	136	7.5
Increased intracranial pressure	120	180	7.8
Release of pressure	156	150	7.4
Increased pressure	132	212	7.8
Increased pressure to point of circulatory failure. Readings 20 sec. before failure	198	210	8.0

DEDUCTIONS.

Certain deductions are admissible from an analysis of the preceding experiments.

1. Alterations in the peripheral circulatory bed, either by the inhalation of amyl nitrite, or by the intravenous injection of histamine, result in a marked reduction in the size of the heart, due, it is suggested, to a decrease in the volume of blood contained in the central circulatory bed incident to a dilatation of the peripheral bed. Gordon and Wells (7) have observed similar variations in heart size in the rabbit and suggested that they were intimately related to blood pressure and made no reference to changes in blood volume distribution. The variations in heart size could not be attributed merely to the altered rate of cardiac contraction (8) since there was no invariable interrelationship established.

2. Contraction of the peripheral vascular bed, either by the intravenous injection of adrenalin, or by an acute increase in the intracranial pressure, results in a demonstrable increase in the size of the heart, due, it is suggested, to an increase in the volume of blood contained in the central circulatory bed. Again, variations in the rate of

cardiac contraction alone cannot be considered responsible for these variations in heart size.

3. The size of the heart is commensurate with the volume flow of blood through it, whether the latter is altered by massive blood letting, by massive transfusions, or by a redistribution of the normal blood volume.

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EXPLANATION OF PLATES.

PLATE 33.

FIG. 1 (see Experiment 2). Changes in size of heart accompanying alterations in blood volume.

FIG. 2 (see Experiment 3). Changes in size of heart following the removal of 800 cc. of blood and its reinfusion 20 minutes later.

FIG. 3 (see Experiment 5). Limiting action of pericardium in acute plethora shown by marked increase in size of heart following incision of the pericardium. (a) Size of heart, pericardium intact, after injection of 750 cc. of whole blood. (b) Size of heart after incising pericardium.

PLATE 34.

FIG. 4. (see Experiment 7). Reduction in size of heart following inhalation of amyl nitrite.

FIG. 5 (see Experiment 9). Changes in size of heart accompanying redistribution of blood volume. (a) Normal. (b) Reduction following injection of histamine. (c) Recovery. (d) Enlargement following injection of adrenalin.

FIG. 6 (see Experiment 11). Marked enlargement of heart accompanying increased intracranial pressure which constricts the peripheral and splanchnic vascular beds, thereby increasing the volume of blood contained in the heart and large central vessels.

FIG. 2.

FIG. 3.

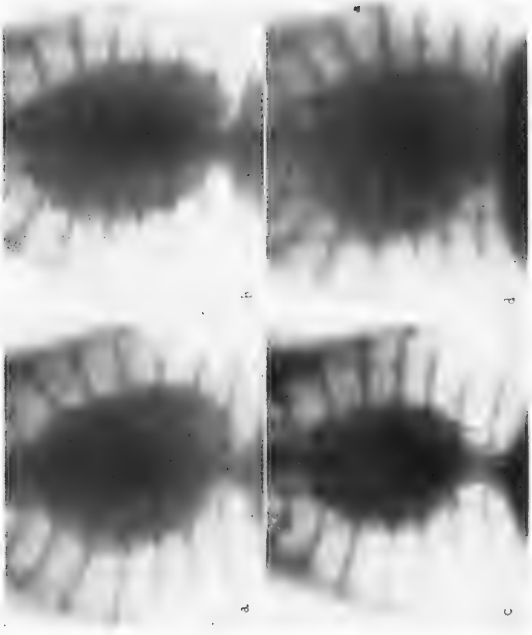
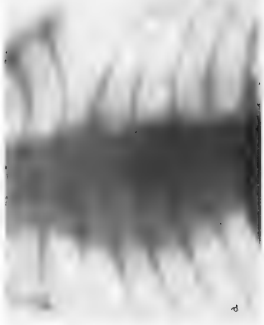


FIG. 1.



(Beck and Holman: Physiological response of circulatory system. II.)

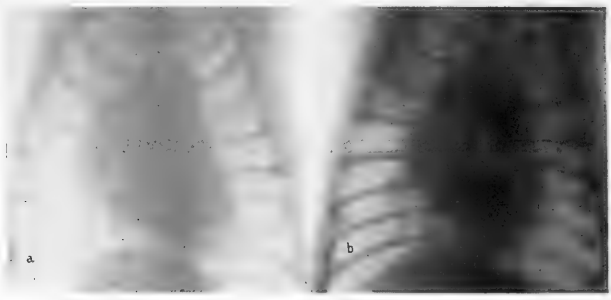


FIG. 4.

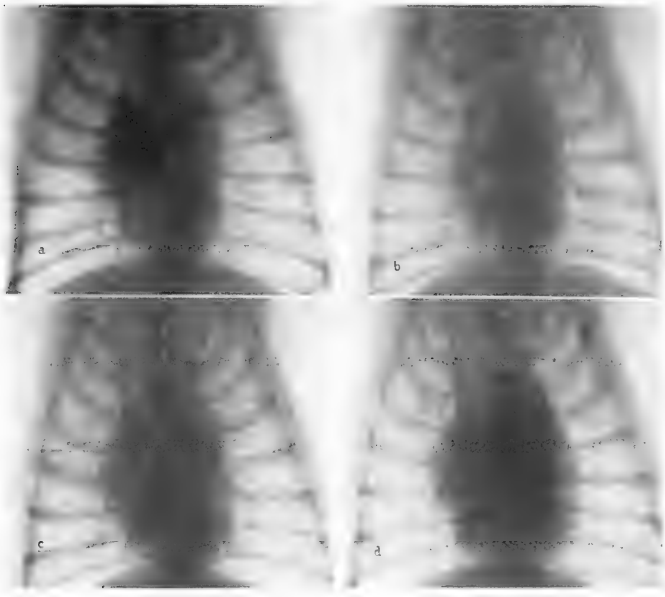


FIG. 5.

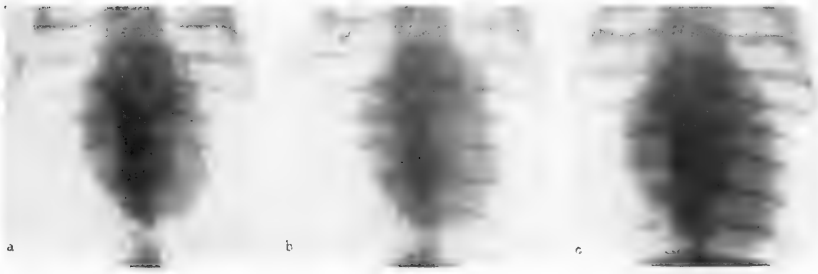


FIG. 6.

(Beck and Holman: Physiological response of circulatory system. II.)

PRIMARY LUNG TUMORS IN MICE FOLLOWING THE CUTANEOUS APPLICATION OF COAL TAR.*

By JAMES B. MURPHY, M.D., and ERNEST STURM.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 35 and 36.

(Received for publication, June 23, 1925.)

It has been a not unusual observation in our experience that mice failing to develop skin cancer as the result of the application of coal tar are found to have tumors of the lungs. The first explanation of the pulmonary tumors which suggests itself is that a cancer of the skin had occurred as a result of the tar, had given off a metastasis to the lung, and had then either healed or sloughed out. But frequent and careful examination of the animals during life failed to show any lesions of the skin suggesting cancer, and at autopsy there were no scars or other evident abnormalities in the painted areas. Furthermore, histological examination of the nodules in the lungs revealed a structure and a type of cell different from the metastases which occur from the tar skin cancers. Spontaneous tumors in the mouse lung have been frequently observed but they rarely occur in such young animals or in such a high proportion of individuals as in our experiments. In the following experiments we have attempted to eliminate completely the probability of skin lesions while determining the influence of the external application of tar on the incidence of primary tumors of the lungs.

Experimental Method.

It is well known that the induction of skin cancer in mice by the application of coal tar requires frequently repeated applications of this agent. With the product used in this laboratory three applications weekly for 3 months suffice to induce cancer in a fair proportion

* This investigation was carried out by means of funds from the Rutherford Donation.

of the animals. When a high rate of incidence is desired it is necessary to continue the paintings for 4 months.

Tar Preparation.—The tar product used throughout these experiments had for its base the residue from a coke oven in which the crude tar had been distilled at a temperature of approximately 377°C.[1] This hard residue was pulverized and extracted with benzene. The soluble fraction was concentrated, washed first with 12 per cent NaOH, and then with 30 per cent H_2SO_4. The precipitates were removed and the benzene fraction was then evaporated down until all of the excess benzene had been removed.[2]

Method of Application.—The tar was applied by means of a swab to an area of skin somewhat less than a centimeter in diameter. There were twelve such areas on the surface of each mouse, painted in rotation according to the following scheme.

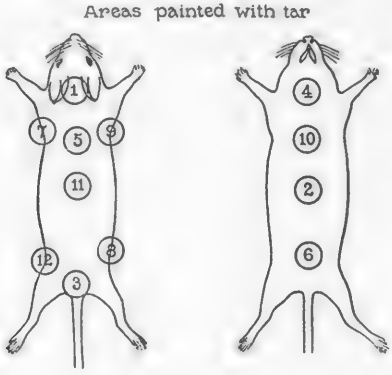
Area.	Days of application of tar.			Area.	Days of application of tar.		
1	1	29	57	7	15	43	71
2	3	31	59	8	17	45	73
3	6	34	62	9	20	48	76
4	8	36	64	10	22	50	78
5	10	38	66	11	24	52	80
6	13	41	69	12	27	55	83

By this method each mouse received thirty-six applications of tar distributed over 83 days. Each individual area received three applications during this time with practically a month between each painting. The selection of the areas and the order in which they were painted were so arranged as to avoid the possibility of overlapping of tarred spots. The location of the areas and the order in which they were painted are shown in Text-fig. 1.

[1] This material was kindly furnished by the Barrett Company.

[2] For details of preparation see Murphy, Jas. B., and Landsteiner, K., *J. Exp. Med.*, 1925, xli, 807.

Experiment 1.—20 mice from 3 to 4 months old, selected from a fairly uniform stock with an extremely low cancer incidence, were painted with tar according to the above scheme. Of these 12 survived for from 1 month to 6 months after the tar paintings had been stopped. When killed 10 of the 12 showed one or more pearly white nodules in the lungs varying in size from 1 to 4 mm. in diameter. There was no detectable change in the skin of the painted regions. Microscopic examination of the lung nodules showed them to be typical epithelial tumors in eight of the specimens. In the other two instances the nodules were lost. The



TEXT-FIG. 1.

lung tumor rate for this experiment was 66.6 per cent, as confirmed by histological examination, and 83.3 per cent if the 2 animals are included in which the diagnosis was made only on the gross specimen.

As a control to this experiment 22 mice from the same stock ranging in age from 8 months to a year, and kept under the same laboratory conditions, were killed. Careful examination of the lungs failed to show a single instance of tumor.

Experiment 2.—A second group of mice was subjected to the same system of tar applications as that used in Experiment 1. All but 5 of the animals were lost in an epidemic early in the experiment. The surviving animals were killed 6 months after the last tar application and 3 of the 5 were found to have tumors of the lungs, confirmed by microscopic examination, and 1 a tumor of the uterus. The tumor incidence for this experiment was thus 80 per cent and there were lung tumors in 60 per cent.

Experiment 3.—This group was made up of 40 mice between 2 and 3 months old, selected from a fairly uniform stock known to have a low tumor incidence. The animals were painted with tar in the same fashion as in Experiment 1. About 4 months after the last application 23 of them were killed. The skin showed no alteration in the painted regions. In 2 animals the lungs were normal but in all of the remaining 21 there were pearly white nodules in these organs. As many as 15 such tumors were found in the lungs of 1 animal. In diameter they varied from 1 to 4 mm. (Text-fig. 2). Microscopic examination showed the growths to be epithelial tumors of the same type as those in the preceding experiments in 18 of the 23 animals. The age of the mice at the time they were killed was between 9 and 10 months.

For controls a group of 16 untreated mice from the same stock, but from 1 to 6 months older than the experimental animals, was kept under the same conditions and killed at the same time. The age of these mice at the time they were killed ranged from 12 to 16 months. In not a single instance was a tumor of the lung or other organ encountered. The tumor incidence for the painted animals was in this experiment 78.3 per cent as compared with 0.0 per cent for the controls.

Character of the Tumors.—The cells making up the tumors are fairly large, cuboidal or ovoid in shape, usually lying in a single layer on either side of a thin shred of stroma (Figs. 1 to 4). The general pattern gives the impression that the tumor is composed of closely packed folds of an epithelial cord. There is some variation in the density of the tumors and in the amount of stroma, but the general histologic type is uniform. Mitotic figures are present in fair number. As a rule the tumors are sharply demarcated from the surrounding lung tissue but it is not uncommon to find finger-like processes invading the lung tissue. Occasionally there is some reaction at the edge of the growth and an inflammatory condition in the neighboring lung tissue but this is an exceptional finding.

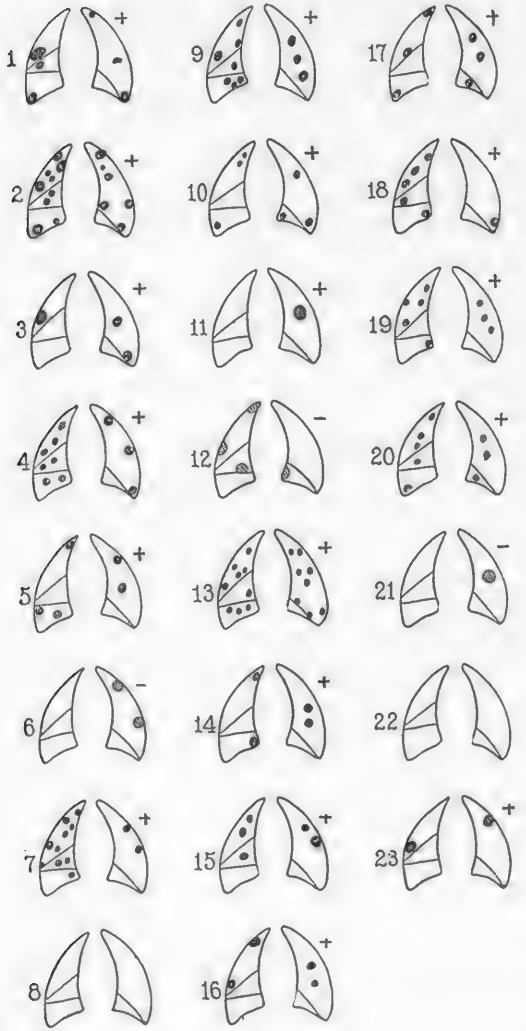
The growths are identical in structure with those described as primary tumors of the lung in mice by Levingood,[3] Haaland,[4] Tyzzer,[5] and Murray.[6] They have been variously designated as adeno-

[3] Livingood, L. E., *Bull. Johns Hopkins Hosp.*, 1896, vii, 177.

[4] Haaland, M., *Ann. Inst. Pasteur*, 1905, xix, 165.

[5] Tyzzer, E. E., *J. Med. Research*, 1907–08, xvii, 155.

[6] Murray, J. A., *3rd Scient. Rep. Imperial Cancer Research Fund*, London, 1908.



TEXT-FIG. 2. Schematic representation of the relative size and number of tumors in the lungs of mice from Experiment 3. The solid black areas indicate that the tumors had the gross appearance of new growths while those cross-hatched were of doubtful nature. The + indicates that a neoplasm was present on microscopic examination and − that no evidence of tumors was found.

carcinoma, papillary cystadenoma, and adenoma. However, judging by the descriptions and published illustrations there is but little variation amongst them and they unquestionably represent a distinct type of epithelial tumor.

DISCUSSION.

As already stated, spontaneous tumors of the lung are not uncommon in certain strains of mice. Tyzzer[5] has reported a high incidence in one of the groups he studied. Slye[7] has noted such tumors in some of her strains. In our tumor stock it is rare to find a lung tumor in animals 12 months old or younger, while the highest rate comes between 26 and 29 months of age.[8] In the case of two special strains, with a lung tumor rate between 59 and 70 per cent, in only one instance has a tumor occurred in a mouse as young as 13 months while the average age is about 20 months. With these facts before us we have attempted to eliminate as completely as possible the chance of spontaneous tumors from our experimental material. In Experiment 1 all but four of the mice were killed before they were 11 months old and the remaining four were killed at 13 months. In Experiment 3 all of the animals were from 9 to 10 months old at the time they were killed and the control mice were from 12 to 16 months old. Therefore it can be definitely said that all of the experimental animals were below the age at which spontaneous lung tumors occur in even a high tumor strain and that they were from a stock showing no lung tumors even at a more advanced age than the experimental animals.

The mechanism of the tar action is not clear. The experiment effectually eliminated the possibility that the lung tumors are in the nature of metastases from skin cancers. Two other possibilities may be considered; *i.e.*, that tar particles get into the lungs through the lymphatics; or that the tar painting so alters the body state that tumors occur at points of incidental irritation at which under ordinary circumstances they would not develop. The first possibility seems a little far fetched. The tar particles might be absorbed through the skin or the alimentary canal since some of the tar is licked off by the animals. In either case the tar would have to pass through at least

[7] Slye, M., Holmes, H. F., and Wells, H. G., *J. Med. Research*, 1914, xxx, 417.
[8] Observations in process of publication.

one set of lymph glands in case it was to reach the lungs. The possibility of the inhalation of particles can be eliminated as the tar is usually either licked or rubbed off before it dries. When some does adhere it becomes very hard and is powdered with difficulty.

In support of the second hypothesis we have accumulated a large amount of data which go to show that the repeated application of tar greatly reduces an animal's resistance to transplanted cancer.[9] Animals of tested resistance, either natural or induced, may be rendered susceptible to a subsequent transplant by tar application. Granting that the application of tar reduces the general resistance to cell growth, is there any evidence that the lungs are the site of irritant processes such as might conceivably act to precipitate the development of neoplasms? Our mice lived in burrows under sawdust and shavings mixed with chopped hay. There is every probability that the inhalation of some small foreign particles resulted in irritation. As a matter of fact inflamed areas are not uncommon findings in the lungs of mice. This hypothesis seems to us the most feasible but further work would be required to establish it.

SUMMARY.

The external application of tar to a number of separated areas on the surface of mice, in such fashion that no single area is irritated sufficiently long to cause lesions of the skin, has resulted in a very high incidence of lung tumors. This incidence ranged from 60.0 per cent in one experiment to 78.3 per cent in another. Control mice from the same stock but from 3 to 6 months older, and for that reason the more liable to spontaneous lung tumors, failed to show a single instance of such growths. Even in a stock in which spontaneous lung tumors had been frequent, the incidence for corresponding age periods has never been above 5.5 per cent while the average has been between 1 and 2 per cent over a period of years. The tumors in the tar-painted animals occur as small white nodules, either single or multiple. They are typical epithelial neoplasms, identical histologically with those described by previous authors as occurring spontaneously in mice.

Some possible factors in the causation of the tumors are briefly discussed.

[9] The data will be published in full in a subsequent article.

EXPLANATION OF PLATES.

PLATE 35.

FIG. 1. Low power photomicrograph of a section through the lower lobe of the lung of a mouse which had received external applications of tar.

FIG. 2. A photomicrograph of an earlier stage of a primary tumor of the lung following external applications of tar.

PLATE 36.

FIG. 3. A primary tumor of the lung following external applications of tar.

FIG. 4. A high power photomicrograph of a lung tumor in a tar-painted mouse, showing mitotic figures.

FIG. 1.

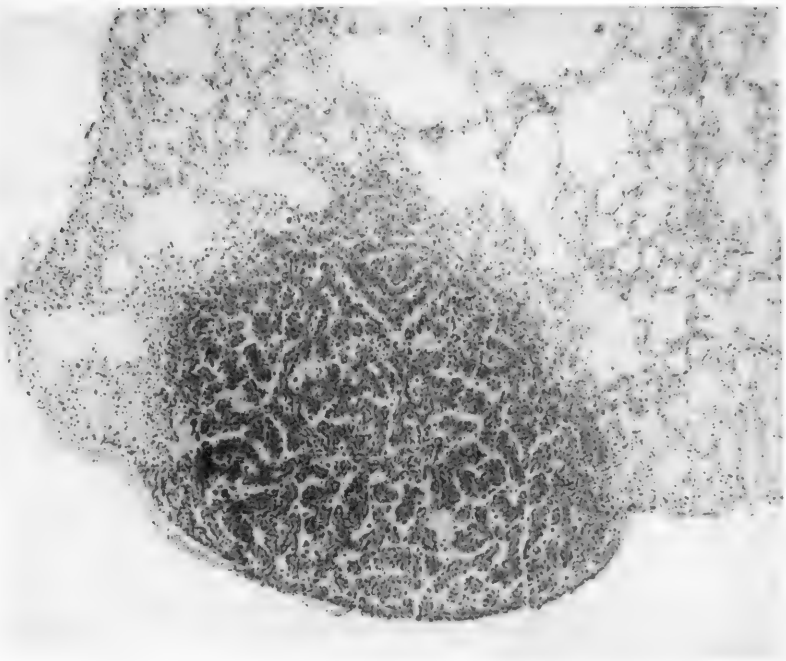


FIG. 2.

(Murphy and Sturm: Primary lung tumors in mice.)

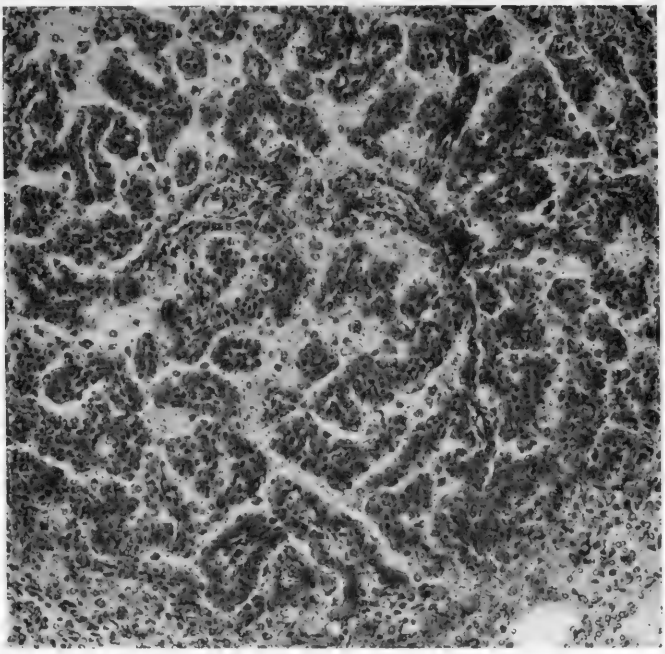


FIG. 3.

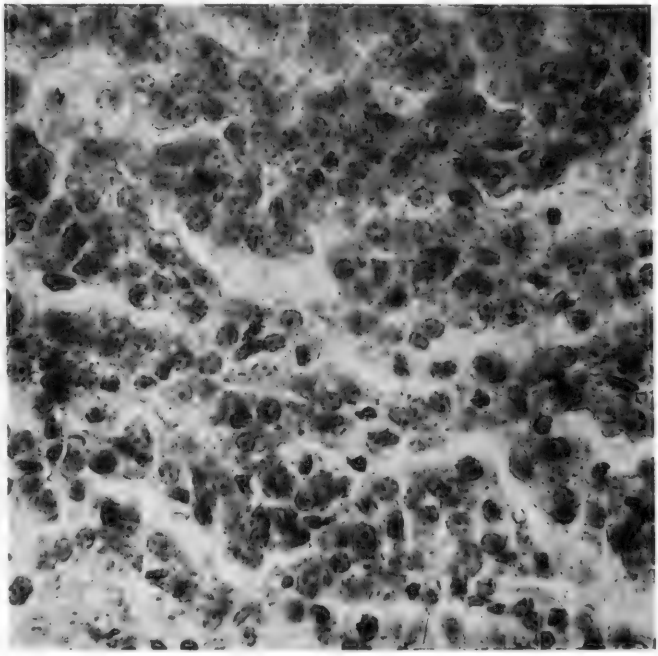


FIG. 4.

(Murphy and Sturm: Primary lung tumors in mice.)

THE SOLUBLE SPECIFIC SUBSTANCE OF A STRAIN OF FRIEDLÄNDER'S BACILLUS.

PAPER I.

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(Received for publication, June 30, 1925.)

In 1921 Toenniessen (1), working on the capsular material of a strain of Friedländer's bacillus, reported the isolation of a snow-white, non-reducing, substantially nitrogen-free polysaccharide which gave a red color with iodine. After hydrolysis the resulting reducing sugars were found to yield an osazone which Toenniessen believed to be that of galactose. These findings were subsequently confirmed by Kramár (2). More recently (3) the so called "soluble specific substance" of the Pneumococcus, first observed by Dochez and Avery (4), has been identified with the polysaccharide portion of the organism. It seemed not unlikely, therefore, that Toenniessen's carbohydrate from the Friedländer bacillus would also possess specific properties, and an investigation of this point was undertaken. Meanwhile the correctness of this surmise has been indicated in a preliminary report by Mueller, Smith, and Litarczek (5), who isolated carbohydrate-containing material with a nitrogen content of 1.3 per cent from a strain of Friedländer's bacillus, and showed that this substance at high dilutions caused precipitation of homologous immune serum.

In the present work a strain of Friedländer's bacillus recovered from a spontaneous guinea pig infection was used. This will be referred to as the E strain, its laboratory designation. From this strain, by a procedure essentially the same as that used in the case of the Pneumococcus (3, 6), a nitrogen-free polysaccharide with specific properties of a most unusual nature has been obtained.

EXPERIMENTAL.

1. Isolation of the Soluble Specific Substance of Friedländer's Bacillus.

Autoclaved washings from 60 to 70 Blake bottles of 48 hour cultures of the E strain of Friedländer's bacillus on solid agar at pH 7.6 were diluted to 3 liters and run through a Sharples centrifuge to remove bacterial debris. The resulting liquid, made slightly alkaline, was treated with 60 gm. of sodium acetate and 4.5 liters of alcohol. After several hours the flocculent precipitate was separated by centrifugation and was dissolved in 2 liters of water. The solution was again centrifuged and reprecipitated as before. With each alkaline precipitation protein material, but no polysaccharide,[1] was eliminated, until finally a biuret-free product was secured. Usually from four to six such precipitations were necessary. At this point the solution of the substance, at a volume of 2 liters, was filtered through a Berkefeld V candle, evaporated to 500 cc., and precipitated with an excess of hot barium hydroxide solution saturated at 60–70°.

The barium salt was suspended in 500 cc. of water and treated with a slight excess of sulfuric acid. The barium sulfate was centrifuged off, washed with very dilute sulfuric acid, and the clear supernatants were neutralized and concentrated to 100 cc. *in vacuo*. The polysaccharide was then precipitated at 0° by the addition of 35 cc. of 1:1 hydrochloric acid and 225 cc. of redistilled alcohol. After standing for 1 hour the precipitate was centrifuged off in the cold, redissolved in 100 cc. of water, and reprecipitated under the same conditions. The final product was washed free from chloride ion with acetone on a hardened filter paper and dried over sulfuric acid *in vacuo*. The yield was 2 to 2.5 gm.

A number of preparations were isolated in this way. The properties of the polysaccharide thus obtained are shown in Table I. The methods of purification employed were the same in all preparations except in the case of Nos. 103 I and 103 II, in which, instead of using agar media, 2 day cultures grown in glucose-aminoid-peptone broth at pH 7.6 were employed. In these cases the initial steps were identical with those employed in the preparation of the soluble specific substances of Types II and III pneumococcus. One volume of alcohol was found to be sufficient for the initial precipitation of the soluble substance. The usual three layer separation was obtained by centrifugation and only the middle layer contained active material. The remainder of the process of purification was as outlined above.

[1] In this preparation complete precipitation of the polysaccharide was followed by tests of portions of the hydrolyzed mother liquors with Fehling solution.

The properties of the various preparations were remarkably uniform, as shown by Table I.

The soluble specific substance of the E strain of Friedländer's bacillus is a white fluffy amorphous powder with acid properties strong enough to turn wet Congo red paper blue when a few particles are dusted upon it. After the substance is dry it dissolves with difficulty in water, but passes readily into solution on neutralization with dilute sodium hydroxide. A 1:200 solution is not precipitated by solutions of silver nitrate, copper sulfate, or phosphotungstic acid,

TABLE I.

Soluble Specific Substance of the Friedländer Bacillus (E.)

Preparation No.	$[\alpha]_D$	Acid equivalent.	Ash.	C	H	N	Percentage of reducing sugars on hydrolysis.	Highest dilution giving a precipitate with immune serum.	
								Anti-Friedländer serum E.	Antipneumococcus serum Type II.
101	+100.0°	670	0.0	44.6*	6.1	0.0	73.0	1:2,000,000	1:2,000,000
103 I	+102.5°	674	0.0			0.0	72.4	1:2,000,000	1:2,000,000
103 II	+100.0°	704	0.0			0.0	70.0	1:2,000,000	1:2,000,000
104	+100.0°	722	0.0			0.06	73.0	1:2,000,000	1:2,000,000
104 A	+100.0°	685	0.0			0.0	72.0		
105 A	+100.0°	706	0.0			0.66	72.0	1:2,000,000	1:2,000,000
105 B†	+101.5°	674	0.0			0.2	73.0		
105 Ad	+100.0°	716	0.0			0.0	78.0		
25 A‡	+ 70.2°	1302	0.35	45.8	6.4	0.0	68.4		1:6,000,000

* Theory for $(C_6H_{10}O_5)_x$, C = 44.4, H = 6.2.

† This represented a residue which failed to pass through a Berkefeld filter.

‡ A preparation of Type II pneumococcus soluble specific substance given for comparison.

but yields precipitates with barium hydroxide and with both neutral and basic lead acetate. It gives no color with iodine-potassium iodide solution. In several of the preparations the polysaccharide was obtained free from nitrogen. When any traces of nitrogen remained these could be removed by the additional methods of purification outlined below. Micro Kjeldahl determinations (Pregl) were made on samples as large as 30 to 40 mg.

In many of its precipitation reactions and in yielding glucose on hydrolysis (see below) the substance resembles the soluble specific

substance of Type II pneumococcus, although in its present state of purity there are points of difference between the two products (compare with 25 A, Table I; also reactions with barium hydroxide and neutral lead acetate). This similarity in chemical properties led to a test of the specific reaction of the substance with Type II antipneumococcus serum. Under these conditions a "specific" immune precipitate resulted, while no reaction occurred in the presence of antipneumococcus sera Types I and III. This unusual phenomenon and its immunological significance are treated in detail in the following paper.

2. Attempts at Further Purification of the Specific Substance.

Since the specific substance yielded a precipitate with uranyl nitrate, a property common to the analogous substances of the three types of Pneumococcus, a portion was precipitated with this salt to see whether additional purification could be effected.

To a neutralized solution of 0.4 gm. of Preparation 104 in 80 cc. of water 20 cc. of 5 per cent uranyl nitrate solution were added. The heavy precipitate which formed was separated after 24 hours by centrifugation. The supernatant fluid gave a negative specificity test and only a faint reaction for uranyl ions. The precipitate was suspended in water and dissolved by addition of the least possible amount of normal hydrochloric acid. Molar potassium dihydrogen phosphate solution was then added until no further precipitate occurred. The uranyl phosphate was centrifuged off and the supernatant liquid, containing the specific substance, was concentrated *in vacuo* to 50 cc. and precipitated by the addition of 1 gm. of sodium acetate and 100 cc. of alcohol. The precipitate was dissolved in 20 cc. of water and the solution cooled to 0° and treated with 3 cc. of hydrochloric acid and 60 cc. of redistilled alcohol. After 1 hour the substance was separated from the slightly active supernatant liquid by centrifugation, redissolved in 20 cc. of water, and thrown into 200 cc. of acetone. The yield was 0.27 gm.

A comparison of this preparation (104 A, Table I) with the original material (104) shows that the only significant change was the removal of the last traces of nitrogen.

The highly adsorbent Alumina A prepared according to Willstätter and Kraut (7) was also used for further purification.

A solution of 0.5 gm. of Preparation 105 A was shaken with 1.5 gm. (calculated as Al_2O_3) of Type A alumina in a volume of 250 cc. at pH 5.0. At the end of 1

hour the alumina, containing the adsorbed polysaccharide, was centrifuged off from the now specifically inactive liquid. The precipitate was extracted once with 250 cc. of N/100 sodium hydroxide for 2 hours and a second time with 200 cc. of N/5 sodium carbonate solution. After the extracts were neutralized with acetic acid they were concentrated *in vacuo* to a volume of 50 cc. and the polysaccharide was precipitated with 100 cc. of alcohol. The specific substance was centrifuged off, dissolved in 25 cc. of water, and the solution was centrifuged from a small amount of insoluble alumina and finally treated at 0° with 5 cc. of 1:1 hydrochloric acid and 60 cc. of redistilled alcohol. After 2 hours the precipitate was centrifuged off, dissolved in 15 cc. of water, and poured into 10 volumes of cold acetone. The product was finally filtered and washed with acetone until free from chloride ion. 0.32 gm. of dry material was recovered.

A comparison of the product (105 Ad, Table I) with the original material (105 A) shows that adsorption and recovery of the polysaccharide had little effect other than elimination of the nitrogen present.

3. Isolation of Glucosazone and Potassium Hydrogen Saccharate from the Hydrolysis Products of the Specific Substance.

1 gm. of Preparation 101 was dissolved in 60 cc. of normal sulfuric acid and the solution was boiled for 5 hours, until no further increase in reducing sugars could be observed. After dilution to 250 cc. the sulfuric acid was quantitatively removed by barium hydroxide solution. The barium sulfate was centrifuged off and the supernatant fluid was concentrated to 30 cc., boiled with Norite, filtered, and finally treated with 1.4 gm. of phenylhydrazine acetate. After heating on the water bath for 1 hour the crystalline osazone which had formed was filtered off. A second fraction was obtained on heating the solution further. Since both portions appeared to be quite pure they were combined, washed with a small amount of methyl alcohol to remove tar, filtered off, and dried. 0.25 gm. of substance was recovered, melting at 201° with decomposition. After recrystallization from 60 per cent ethyl alcohol the decomposition point was 203–204°. A mixture of this compound with the osazone prepared from pure glucose also melted at 203–204°.

0.1000 gm. of substance gave 13.40 cc. of N_2 at 23°C., 761.7 mm.

Calculated for $C_{18}H_{22}O_4N_4$; N, 14.45 per cent. Found: N, 14.49 per cent.

0.0576 gm. of the compound dissolved in 5 cc. of pyridine-alcohol mixture had an initial $[\alpha]_D$ of $-67.9°$ which decreased after standing 4 days to $-24.2°$.

From the analysis, melting point, and direction of mutarotation (8) the substance is undoubtedly glucosazone. The identification of glucosazone limits the sugar formed on hydrolysis of the specific substance either to glucose, fructose, or mannose.

In order to ascertain which of these sugars was actually present 1 gm. of Preparation 103 II was boiled for 5½ hours under a reflux condenser with 100 cc. of one-half normal nitric acid. The hydrolysis products had a specific rotation of +75°. The solution was concentrated *in vacuo* to 5 cc. and to it was added 1 cc. of concentrated nitric acid. After 24 hours at room temperature the mixture was boiled for 2.5 minutes and then quickly evaporated, with stirring, on a large watch-glass over a boiling water bath. A thick paste was obtained which was twice evaporated with a small quantity of water, to expel the last traces of nitric acid, and was finally dissolved in 5 cc. of water. The solution was made strongly alkaline with 40 per cent potassium hydroxide solution and then acidified with glacial acetic acid. After 5 hours the crystals of potassium acid saccharate which had separated were filtered off and recrystallized from 2 cc. of boiling water.

0.0494 gm., ignited with sulfuric acid, yielded 0.0173 gm. K_2SO_4.

Calculated for $COOH(CHOH)_4COOK$; K, 15.75 per cent. Found: 15.70 per cent.

It is thus evident that as in the case of the specific substance of the Type II pneumococcus glucose is the principal sugar from which the specific substance of Friedländer's bacillus (Strain E) is built up.

DISCUSSION.

It is clear from the foregoing that the E strain of Friedländer's bacillus yields, on fractionation, a nitrogen-free polysaccharide with specific properties of the order possessed by the soluble specific substances of the three fixed types of Pneumococcus. It is a strong acid with an equivalent value of about 685, sparingly soluble in water after drying, but yielding soluble alkali salts. The specific optical rotation is +100°. The polysaccharide itself is non-reducing, but on hydrolysis with mineral acid yields reducing sugars, among which glucose has been shown to be present. In the present instance, as in those of the pneumococci, carbohydrate and specific function are apparently inseparable, and the isolation of a fourth specific substance of this nature adds additional weight to the growing mass of evidence that the soluble specific substances of microorganisms are often actually polysaccharides (*cf.*(5) also Mueller and Tomcsik (9)[2]).

[2] These investigators isolated specific polysaccharide material from Friedländer's bacillus and yeast. Since the present communication was accepted for publication Laidlaw and Dudley (*Brit. J. Exp. Path.*, 1925, vi, 197) have described a specifically precipitating, non-nitrogenous carbohydrate isolated from tubercle bacilli.

While the specific substance of the E strain, as purified up to the present, has properties which set it apart from the three analogous substances of the fixed types of Pneumococcus there is nevertheless a certain resemblance to that of the Type II pneumococcus, a resemblance extending even to precipitation with Type II antipneumococcus serum. The immunochemical relationships of these otherwise widely different microorganisms are treated in the succeeding paper.

Work on the specific substance of the E strain is being continued and the soluble specific substances of other strains of the Friedländer bacillus are also under investigation.

SUMMARY.

1. A method is given for the isolation of a specifically reacting nitrogen-free polysaccharide from the so called E strain of Friedländer's bacillus.

2. The properties of this polysaccharide are described.

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THE SOLUBLE SPECIFIC SUBSTANCE OF FRIEDLÄNDER'S BACILLUS.

Paper II. Chemical and Immunological Relationships of Pneumococcus Type II and of a Strain of Friedländer's Bacillus.

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(Received for publication, June 30, 1925.)

The preceding paper (1) records the initial attempts to isolate and purify the soluble specific substance of the so called E strain of a bacillus of the Friedländer group. The organism was originally isolated from a spontaneous epidemic of pneumonia among stock guinea pigs. The specifically reactive substance which has been isolated appears to be a nitrogen-free polysaccharide differing in many respects from the soluble specific substances recovered from the three fixed types of Pneumococcus (2). However, as in the case of the soluble specific substance of Pneumococcus Type II, only glucose could readily be identified among the products of hydrolysis. Moreover, the percentage of reducing sugars on hydrolysis is almost the same in both substances, and it therefore seemed possible that both of them might in reality be identical and that the differences observed might be due to impurities. In the preceding paper procedures were outlined which were designed to eliminate any impurities present in the Friedländer polysaccharide, but the employment of these methods only resulted in recovery of the original substance practically unaltered.

A comparison of the specific substance obtained from Type II pneumococcus with that isolated from the E strain of Friedländer's bacillus shows that both polysaccharides rotate the plane of polarized light to the right. In the case of the Friedländer substance the specific rotation is $+100°$, while the Type II pneumococcus sub-

stance rotates the plane of polarized light about +74°. Both substances have acidic properties, but the Friedländer specific substance has an acid equivalent approximately one-half that of the Type II polysaccharide. Neither of the two products gives glucuronic acid tests as do the specific substances of Types I and III pneumococcus. Both polysaccharides fail to form precipitates when treated with solutions of silver nitrate, copper sulfate, or phosphotungstic acid; both are precipitated by solutions of uranium nitrate and basic lead acetate. Whereas the Type II pneumococcus specific substance gives no precipitate with either barium hydroxide or neutral lead acetate, the Friedländer polysaccharide is precipitated by both of these reagents.

Because the two specific substances, although of widely different biological origin, resemble each other so closely in some of their chemical properties, the Friedländer polysaccharide was tested with Type II antipneumococcus serum, and a precipitin reaction was found to occur. On the other hand, there was absence of precipitation when this substance was tested with antipneumococcus serum of the other two fixed types. It then became necessary to determine as far as possible the immunological relationships not only of the soluble substances of the E strain of the Friedländer bacillus and of Type II pneumococcus, but of the microorganisms themselves. The present paper deals with the facts so far ascertained in this study.

EXPERIMENTAL.

Microorganisms.—The strain of Gram-negative bacillus from which the specific polysaccharide was derived corresponds in its cultural and biological reactions to organisms of the *encapsulatus* group, and for laboratory purposes is referred to as the E strain. Five other strains, four of which, as will be shown later, are similar in their serological reactions to the original organism, have been used in this study. It does not seem essential at this time to enter into a detailed description of the individual cultural characteristics of each strain;—they all possess in common the property of luxuriant growth on ordinary media with the elaboration of the abundant gummy, mucoid material characteristic of the group.

Methods.—Rabbits were immunized by the intravenous injection of suspensions of heat-killed organisms; *small* doses were administered every day for 6 days, and they were repeated after an interval of a week. Three courses of injections were given, and the animals were bled 9 days following the last inoculation.

The agglutination and precipitin tests were done as previously described for Pneumococcus. The protective power of type sera against Friedländer's bacilli and pneumococci was tested by injecting white mice intraperitoneally with varying doses of the virulent organisms and at the same time injecting a fixed quantity (0.2 cc.) of immune serum. All animals were observed for at least 10 days following injection, and those surviving this period were considered effectively protected.

Agglutination.—Immune rabbit sera were prepared against two strains of Friedländer's bacillus. One of these was the culture E from which the specific polysaccharide was isolated. The second culture (Sc) was isolated from the blood of a patient suffering from pneumonia. The study of the agglutinin reactions of these two immune sera afforded an opportunity, first, to establish the specific relationship of these two strains to each other and to other organisms of the group; and second, to confirm the immunological relationships between Type II pneumococcus and the E strain.

It immediately becomes evident from Table I that, as others have previously observed, the *encapsulatus* group does not consist of organisms which are immunologically identical. The two strains (E and Sc) chosen at random are antigenically distinct one from the other. Observations by Julianelle (3) indicate, however, that these two strains are representatives of two distinct but not unusual immunological types, since of forty strains of Friedländer's bacillus which he has tested, seventeen were found to be immunologically identical with either Strain E or Strain Sc. It is not the purpose of this paper, however, to deal with the serological relationships within the Friedländer group. Of more immediate interest is the fact, as shown by the experiments recorded in Table I, that the Friedländer Type E strain reacts in antipneumococcus Serum II and that Type II pneumococcus is agglutinated in serum produced by immunization with the E strain of Friedländer's bacillus. An immunological similarity, therefore, exists between these two biologically remote organisms, and this relationship is reciprocal. The experiment also shows that there is no immunological relationship between Pneumococcus Type II and the other strain of Friedländer's bacillus tested (Sc), which, although a typical organism of the *encapsulatus* group, is immunologically distinct from Strain E.

TABLE I.

Agglutination Reactions of Two Strains of Friedländer's Bacillus and Pneumococcus Type II in Homologous and Heterologous Sera.

Culture.	Immune sera.														
	Anti-Friedländer Sc.*					Anti-Friedländer E.†					Antipneumococcus Type II.‡				
	1:5	1:10	1:20	1:40	1:80	1:5	1:10	1:20	1:40	1:80	1:10	1:20	1:40	1:80	1:160
Friedländer's bacillus (Sc)....	++++	++++	+	−	−	−	−	−	−	−	−	−	−	−	−
Friedländer's bacillus (E).....	−	−	−	−	−	++++	++++	++++	++	−	+++	+++	+	−	−
Pneumococcus Type II.....	−	−	−	−	−	++++	++++	++	++	−	++++	++++	++	++	±

++++ indicates complete, compact, disk-like agglutination; +++, marked, compact, disk-like agglutination; ++, clumping—more easily broken up; +, partial clumping; ±, slight clumping; −, negative.

* Rabbit 77 D.

† Rabbit 84 D.

‡ Horse 91 A.

That other strains of Friedländer's bacilli, which are immunologically identical with Strain E, also react with antipneumococcus serum Type II just as does the E strain is shown in Tables II, III, and IV, in which are presented the results of agglutination reactions of six

TABLE II.

Agglutination of Friedländer's Bacillus and Pneumococcus Type II in Anti-Freidländer Sera.

Friedländer's bacillus.	Anti-Friedländer Serum E.					Anti-Friedländer Serum Sc.				
	1:5	1:10	1:20	1:40	1:80	1:5	1:10	1:20	1:40	1:80
E	++++	++++	++++	+++	−	−	−	−	−	−
K	++++	++++	+++	+++	−	−	−	−	−	−
H_1	++++	++++	++++	++	−	−	−	−	−	−
H_2	++++	++++	++++	+++	−	−	−	−	−	−
F_6	++++	++++	++++	++++	++	−	−	−	−	−
Sc	−	−	−	−	−	++++	++++	+	−	−
Pneumococcus Type II.	+++	+++	++	±	−	−	−	−	−	−

TABLE III.

Agglutination of Friedländer's Bacillus in Antipneumococcus Serum Type II.

Friedländer's bacillus.	Antipneumococcus serum.						Normal horse serum.		Salt solution.
	Type I.		Type II.		Type III.				
	1:1	1:10	1:1	1:10	1:1	1:10	1:1	1:10	
E	−	−	++++	++++	−	−	−	−	−
K	−	−	++++	++++	−	−	−	−	−
H_1	−	−	++++	++++	−	−	−	−	−
H_2	−	−	++++	++++	−	−	−	−	−
F_6	−	−	++++	++++	−	−	−	−	−
Sc	−	−	−	−	−	−	−	−	−
G_9	−	−	−	−	−	−	−	−	−

additional strains. When the strains are immunologically related to Strain E they react with antipneumococcus serum Type II; when they are immunologically distinct (Sc and G_9, Table III) they fail to react. Moreover, Table III shows that the agglutination of Friedländer's bacilli of the Strain E group does not occur with anti-

pneumococcus sera of Types I and III, but only in that of Type II. This fact suggests that the immunological relationship between certain strains of the Friedländer bacillus and certain strains of Pneumococcus is dependent upon chemical similarities in the soluble specific substances of the organisms concerned. Table IV shows that while Friedländer strains of the E type all react about equally in Type II antipneumococcus serum, they do not react in as high dilution as does Pneumococcus Type II itself. This difference in the capacity of the two kinds of bacteria to react to the same degree in the same immune serum shows a lack of complete immunological identity between them, a fact presumably related to the minor chemical

TABLE IV.

Agglutination of Friedländer's Bacillus by Antipneumococcus Serum II.

Friedländer's bacillus (strains).	Antipneumococcus serum Type II.							Control.
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	
E	++++	++++	+++	+	−	−	−	−
K	++++	++++	++++	±	−	−	−	−
H_1	++++	++++	+++	±	−	−	−	−
H_2	++++	++++	++++	+++	−	−	−	−
F_6	++++	++++	++++	±	−	−	−	−
Sc.	−	−	−	−	−	−	−	−
G_9	−	−	−	−	−	−	−	−
Pneumococcus Type II.	++++	++++	++++	++++	++++	++	−	−

differences which appear to exist between the soluble specific substances of the two organisms.

Agglutinin Absorption.—Krumwiede and his associates (4) have recently published a critical and analytical review of the subject of agglutinin absorption in which they point out the significance of the method for bacterial identification. It will be important to apply this method with great exactness to the present problem, but at present we can only record the results so far obtained.

The results of observations as given in Tables V and VI indicate the failure of reciprocal absorption. Unabsorbed anti-Friedländer serum which agglutinates both the Strain E Friedländer bacillus and Pneumococcus Type II to about the same titer, loses its agglu-

TABLE V.

Agglutinin Absorption.

Culture.	Anti-Friedländer Serum E.												Normal rabbit serum control.			
	Unabsorbed.				Absorbed with Friedländer's bacillus (E).				Absorbed with Pneumococcus Type II.							
	1:5	1:10	1:20	1:40	1:5	1:10	1:20	1:40	1:5	1:10	1:20	1:40	1:5	1:10	1:20	1:40
Friedländer's bacillus (E)	++++	++++	+++	++	−	−	−	−	++++	++++	+++	+	−	−	−	−
Pneumococcus Type II	++++	+++	+++	+	−	−	−	−	−	−	−	−	−	−	−	−

++++ indicates complete, compact agglutination; −, no reaction.

TABLE VI.

Agglutinin Absorption.

Heat-killed suspension of	Anti-Friedländer Serum E.											
	Unabsorbed.						Absorbed by Pneumococcus II.					
	1:5	1:10	1:20	1:40	1:80	1:160	1:5	1:10	1:20	1:40	1:80	1:160
Pneumococcus Type II	++++	++++	++++	+±	−	−	−	−	−	−	−	−
Friedländer's bacillus (E)	++++	++++	+++	+	−	−	++++	++++	+±	±	−	−

Heat-killed suspension of	Antipneumococcus serum Type II.															
	Unabsorbed.								Absorbed with Friedländer's bacillus (E).							
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640
Pneumococcus Type II	++++	++++	++++	+++	++±	++	+	−	++++	++++	++++	+++	++	+	±	−
Friedländer's bacillus (E)	++++	++++	+	±	−	−	−	−	−	−	−	−	−	−	−	−

tinins for both organisms when absorbed with the homologous strain of Friedländer's bacillus. On the other hand, absorption of the same serum with Pneumococcus Type II removes only the agglutinins for the absorbing organism and leaves the antibodies for the Friedländer strain only slightly reduced. Similarly, absorption of antipneumococcus serum Type II with Pneumococcus of the homologous type results in complete removal of agglutinins for both organisms, while absorption of the same serum with the E strain of Friedländer's bacillus takes out the antibodies for this organism and only slightly reduces the agglutinating power of the serum for Type II pneumococcus.

TABLE VII.

Precipitin Reactions: Anti-Friedländer Serum E against Carbohydrate Fraction Derived from Homologous Strain.

Anti-Friedländer Serum E.	Carbohydrate of Friedländer's bacillus (E).						
	1:50,000	1:100,000	1:250,000	1:500,000	1:1,000,000	1:2,000,000	1:4,000,000
0.2 cc.							
Rabbit 84	++++	++++	+++	+++	++	+±	±
" 83	++++	++++	+++	+++	+±	+	−
" 82	++++	++++	+++	++	+±	+	−
" normal	−	−	−	−	−	−	−
Antipneumococcus serum Type II	+++	+++	+++	+++	+++	++	±

Precipitin Reactions.—Immunization of rabbits with Friedländer's bacillus (Strain E) engenders antibodies which not only agglutinate organisms of the same type, but which precipitate the soluble specific polysaccharide derived from the bacterial cell. As in the case of the specific polysaccharide of Pneumococcus the reactivity of the Friedländer substance is exhibited in dilutions as great as 1:2 million. This fact is evident in Table VII, in which immune rabbit sera in quantities of 0.2 cc. are shown to react with minute amounts of the carbohydrate derived from the homologous strain of Friedländer's bacillus. Just as antipneumococcus serum has been found to agglutinate Friedländer's bacilli of the type represented by Strain E, so also it reacts in precipitin tests with the Friedländer specific substance in high dilution.

Cross-precipitin reactions between the pneumococcus and Friedländer polysaccharides are shown in Table VIII. The soluble specific substances of both organisms are reciprocally reactive with the antibacterial serum of each in dilutions of 1:2 million. The physical properties of the immune precipitate formed by the union of the pneumococcus carbohydrate with the anti-Friedländer serum differ from those of the opaque disk characteristic of the other reactions in that the precipitate is less heavy, more transparent, and tends to form a gelatinous film.

The "specificity" of the reaction is illustrated in Table IX. Here, four protein-free carbohydrates prepared from four different organ-

TABLE VIII.

Cross-Precipitin Reactions.

Immune sera.	Carbohydrate of Friedländer's bacillus (E).					Carbohydrate of Pneumococcus Type II.				
	1:200,000	1:500,000	1:1,000,000	1:2,000,000	1:4,000,000	1:200,000	1:500,000	1:1,000,000	1:2,000,000	1:4,000,000
Anti-Friedländer E. Rabbit 84.....	++++	+++	++	+	±	++++*	++	+	±	−
Antipneumococcus Type II. Horse 91 A.....	++++	+++	++	+	−	++++	+++	+	±	−

* Thin translucent film-like precipitate.

isms are tested in cross-precipitin reactions with the corresponding antibacterial serum of each strain. The three polysaccharides derived from the three fixed types of Pneumococcus show only the specific type reactions in antipneumococcus serum. The cross-precipitation between the Type II pneumococcus specific substance and the anti-Friedländer serum, and the reverse reactions are again evident.

Precipitin Absorption.—Aliquot portions of diluted anti-Friedländer serum were separately absorbed with the homologous culture and with Pneumococcus Type II. The absorbed antibacterial serum was then tested for precipitins against dilutions of the carbohydrates derived from each of the organisms. The results are given in Table X.

As in the case of agglutinin absorption, the anti-Friedländer serum (E) treated with the homologous bacilli loses its precipitins for both

TABLE IX.

Precipitin Reactions of Soluble Specific Substances of Pneumococcus Types I, II, III, and of Friedländer's Bacillus (Strain E).

Immune sera.	Carbohydrate fraction isolated from‡			
	Pneumococcus.			Friedländer's bacillus (E).
	Type I.	Type II.	Type III.	
Antipneumococcus*Type I.....	++++	−	−	−
" " II.....	−	++++	−	++++
" " III.....	−	−	++++	−
Anti-Friedländer† E.....	−	++	−	++++

− indicates no reaction; ++, delicate film-like scale; ++++, heavy, compact, disk-like precipitate.

* Immune horse serum.

† Immune rabbit serum.

‡ Isolated, purified soluble specific substance in dilution of 1:50,000.

TABLE X.

Precipitin Absorption.

Carbohydrate fraction of		Anti-Friedländer Serum E.			Control.
		Unabsorbed.	Absorbed with Friedländer's bacillus (E).	Absorbed with Pneumococcus II.	Normal rabbit serum.
Friedländer's bacillus (E)	1:10.000	+++	−	++	−
	1:50,000	++++	−	+++	−
	1:100,000	++++	−	+++	−
	1:200,000	++++	−	++++	−
Pneumococcus Type II.....	1:10,000	+	−	−	−
	1:50,000	++	−	−	−
	1:100,000	++±	−	−	−
	1:200,000	+++	−	−	−

the Friedländer and Pneumococcus II specific substances, whereas absorption with Type II pneumococci removes the antibodies for the pneumococcus polysaccharide, but leaves those for the Fried-

länder substance only slightly diminished. The converse of this, in which antipneumococcus serum is absorbed with each of the two cultures, yields similar results, Pneumococcus Type II removing the precipitins for both specific substances and the Friedländer bacillus depleting the serum only of antibodies for its own substance.

Protection.—The facts brought out by the test-tube reactions of agglutination and precipitation find added confirmation in the more final proof of reciprocal protection against infection in the animal body.

The six strains of Friedländer's bacilli tested for virulence in mice have each caused a fatal infection in doses as small as 1 ten-millionth cc. The protective power of immune sera against Friedländer's bacilli and pneumococci was tested by injecting white mice intraperitoneally with increasing doses of virulent organisms together with a fixed amount of type serum. Animals surviving 10 days were considered effectively protected. All animals except the virulence controls received 0.2 cc. of immune serum.

The serum of a rabbit immunized with the Friedländer bacillus (Strain E) was first tested for its power to protect mice against infection with virulent cultures of homologous and heterologous types of the Friedländer bacillus.

The protocol given in Table XI shows that 0.2 cc. of the anti-Friedländer serum Type E protected animals against 0.1 cc. of a virulent culture of the homologous strain which without immune serum caused death of the mice in doses of one-millionth cc. On the other hand, the same serum afforded no protection in mice against a virulent heterologous Strain Sc, which, by the reaction of agglutination, was shown to be of another type.

Since the E strain of Friedländer's bacillus was found to be agglutinated and its soluble specific substance to be precipitated by antipneumococcus serum Type II, it was of interest to determine whether protection against infection with a biologically different organism was possible by the use of antipneumococcus serum Type II. The results of this experiment are given in Table XII.

It is evident from Table XII that mice inoculated with 0.2 cc. of antipneumococcus serum Type II were effectively protected against at least a thousand lethal doses of the virulent E strain of Friedländer's bacillus. As in the agglutinin and precipitin tests the

"specific" nature of this protective reaction is shown by the fact that antipneumococcus serum Type I afforded no protection whatever against infection (Table XIII).

TABLE XI.

Protective Action of Anti-Friedländer Serum (E) against Friedländer's Bacillus of Homologous and Heterologous Types.

Anti-Friedländer Serum E.	Friedländer's bacillus.			
	Strain E.		Strain Sc.	
	Amount of culture.	Result.	Amount of culture.	Result.
cc.	*cc.*		*cc.*	
0.2	0.1	S.	0.1	D. 17
0.2	0.01	"	0.01	" 18
0.2	0.001	"	0.001	" 22
0.2	0.0001	"	0.0001	" 22
0	0.00001	D. 40	0.00001	" 30
0	0.000001	" 18	0.000001	" 23

In this and the following tables S. indicates survived; D., death, the numerals representing the hours before death of the animal occurred.

TABLE XII.

Protective Action of Antipneumococcus Serum Type II against Friedländer's Bacillus (E).

Friedländer's bacillus (E).	Antipneumococcus Serum II.		Virulence controls.
	Amount.	Result.	
cc.	*cc.*		
0.001	0.2	S.	
0.0001	0.2	"	
0.00001	0.2	"	
0.000001	0.2	"	
0.0000001	0.2	"	
0.00001	0		D. 51
0.000001	0		" 41
0.0000001	0		" 44

The anti-Friedländer serum prepared by immunization with Strain E protected mice against a hundred thousand lethal doses of another strain (K), which, by agglutination, was classified as belonging to the same type as E. Similarly antipneumococcus serum

Type II afforded protection against the K strain just as it did against the E strain; a fact which emphasizes again the immunological similarity of Friedländer's bacillus of this type to Type II pneumococcus. The same protocol (Table XIII) shows that antipneumococcus serum Type I is wholly without protective action against this type of Fried-

TABLE XIII.

Protective Action of Antipneumococcus Serum against Friedländer's Bacillus (K).

Friedländer's bacillus (K) (Type E).	Antipneumococcus sera.		Anti-Friedländer Serum E.	Virulence controls.
	Type I. 0.2 cc.	Type II. 0.2 cc.	Rabbit 84. 0.2 cc.	
0.1		D. 42	D. 18	
0.01		" 18	S.	
0.001	D. 18	S.	"	
0.0001	" 18	"	"	
0.00001	" 42	"	"	D. 21
0.000001	" 18			" 42
0.0000001				" 19

TABLE XIV.

Protective Action of Anti-Friedländer Sera against Pneumococcus Type II.

Pneumococcus Type II culture.	Virulence controls.	Anti-Friedländer serum.		Antipneumococcus serum Type II (Horse 91 A). 0.2 cc.
		Rabbit 84 D immunized with Strain E. 0.2 cc.	Rabbit 77 D immunized with Strain Sc. 0.2 cc.	
cc.				
0.2		D. 46	D. 20	D. 42
0.1		" 46	" 20	" 72
0.01		S.	" 20	S.
0.001		"	" 20	"
0.0001		"	" 26	"
0.00001	D. 36			
0.000001	" 46			
0.0000001	" 46			

länder's bacillus. Moreover, Table XIV brings out the fact that protective power against Type II pneumococcus infection is not possessed by the anti-Friedländer serum produced by Strain Sc, which both by agglutination and protection has been shown to belong to a type different from the effective Type E strains.

The further data presented in Tables XIV, XV, and XVI demonstrate the comparable protective power of antipneumococcus Type II and the anti-Friedländer E sera against infection with virulent Type

TABLE XV.

Protective Action of Anti-Friedländer Serum E against Pneumococcus Type II.

Pneumococcus Type II culture.	Immune sera.				Virulence controls.
	Anti-Friedländer E (Rabbit 84).		Antipneumococcus II (Horse 91 A).		
	Amount.	Result.	Amount.	Result.	
cc.	cc.		cc.		
0.2	0.2	D. 19	0.2	D. 24	
0.1	0.2	S.	0.2	" 48	
0.01	0.2	"	0.2	S.	
0.001	0.2	"	0.2	"	
0.00001	0		0		D. 20
0.000001	0		0		" 20
0.0000001	0		0		" 36

0.0000001 cc. of this culture = 450 colonies.

TABLE XVI.

Comparative Protective Value of Anti-Friedländer and Antipneumococcus Serum against Pneumococcus Type II.

Culture Pneumococcus Type II.	Immune sera.				Virulence control.
	Anti-Friedländer E (Rabbit 84).		Antipneumococcus Type II (Horse 91 A).		
	Amount.	Result.	Amount.	Result.	
cc.	cc.		cc.		
0.2	0.2	S.	0.2	D. 44	
0.1	0.2	"	0.2	" 20	
0.01	0.2	"	0.2	" 44	
0.001	0.2	"	0.2	S.	
0.0001	0.2	"	0.2	"	
0.00001	0		0		D. 26
0.000001	0		0		" 26
0.0000001	0		0		" 44

II pneumococci. Indeed the protective potency of the anti-Friedländer serum in pneumococcus infection in two of the three experiments is greater than, and in the third test, equal to that exhibited

by the Pneumococcus Type II immune serum itself. This is all the more striking since in the former case immune rabbit serum was used and in the latter the serum of a horse which had received more intensive immunization.

The peculiar "specificity" of the reciprocal protection of antisera of two kinds of bacteria so widely different in other biological characters is confirmatory of the immunological relationships of these organisms brought out by the cross-reactions of agglutination and precipitation.

DISCUSSION.

While comparison of the chemical properties of the two soluble specific substances isolated from Pneumococcus Type II and Friedländer's bacillus (Strain E) reveals many points of resemblance, differences are also found which are too great to be ignored. That the substances in reality are identical and that the observed differences depend only upon impurities present may possibly be the case, but the evidence so far obtained is entirely opposed to this assumption. It has been pointed out in the preceding paper (1) and in the papers dealing with the soluble specific substances of pneumococci (2, 5) that widely differing methods of preparation, calculated to remove different kinds of accompanying inert matter, have yielded strictly comparable products. This fact cannot be taken to indicate that the specific substances as at present isolated are pure chemical compounds, but it at least makes reasonable the assumption that in each instance a large proportion of the adventitious impurities has been eliminated. The view that the specific substance isolated from Pneumococcus Type II and that recovered from the Friedländer bacillus (Strain E) are not identical is further supported by certain of the serological findings, especially those which show that the absorption of agglutinins and precipitins is not reciprocal with the two organisms. If the fact that bacteria possess mutual absorptive capacity be accepted as the criterion of their antigenic identity then the failure of the organisms in question to exhibit this property may be taken as further evidence of the lack of identity of the substances involved.

However, granted a chemical difference between the two specific substances, it becomes necessary to account for their marked im-

munological similarity. In the absence of further evidence as to the structural relations of the two substances, which can only be obtained when large amounts of material become available, it seems reasonable to assume that both contain in a portion of the complex molecule the same or a closely similar configuration of atoms. This essential similarity in molecular grouping would then determine the immunological similarity of the two substances.

In the case of Pneumococcus it has been shown that the polysaccharides by themselves are not antigenic, and it is believed that they become antigenic only when attached to some other substance, possibly the protein of the cell. The type-specific character of the antigenic response, however, is dependent almost entirely upon the nature of the polysaccharide and not upon the substance to which it is attached. Therefore, since the specific carbohydrate substance of the Friedländer bacillus (Strain E) and that of Type II pneumococcus possess in common similar chemical properties, the antigenic response to each may also be similar even though the proteins or other substances with which they are combined be quite dissimilar. A discussion of the actual number of antigens and antibodies present must be deferred until more facts are available (*cf.*, however, in this connection, Landsteiner and van der Scheer (6)).

A striking and probably analogous example of common antigenic properties in substances of remote biological origin is furnished by the phenomenon of heterogenetic specificity originally described by Forssman (7). This investigator showed that following the injection of animal tissues of unrelated species common hemolytic antibodies for sheep corpuscles appear. Landsteiner (8) and Taniguchi (9) have shown that such heterogenetic antigens consist of two component parts, one a protein, the other probably a lipoid substance. Landsteiner and Simms (10) have found that the lipoid constituent, although itself practically devoid of antigenic properties, acquires true antigenicity when combined with protein, and that the antibodies thus induced react with the isolated lipoid fraction.

The fact that two biologically unrelated organisms, Pneumococcus Type II and Friedländer's bacillus (Strain E), possess certain similar serological and antigenic properties suggests that examples of heterogenetic specificity likewise occur among bacteria. In the case

of bacteria, however, the specific substance involved instead of being a lipoid appears to be a polysaccharide. From the results reported in the present papers it further appears probable that when the analogous specific polysaccharides of otherwise totally unrelated microorganisms correspond sufficiently in chemical constitution an immunological correspondence also results. This type of immunological correspondence in no way invalidates the systematic classification of bacteria based upon the more usual and general methods of species determination. It is of greater immediate significance in connection with the study of problems dealing with bacteria as disease-producing agents than in the study of bacteria in their genetic relationships.

SUMMARY.

The chemical and immunological properties of the soluble specific substances of a strain of Friedländer's bacillus and Pneumococcus Type II are described and correlated, and the serological and antigenic similarity of these biologically unrelated organisms is discussed as an example of heterogenetic specificity among bacteria.

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THE SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS.

Third Paper.

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In the preceding papers of this series (1) the significance of the so called "soluble specific substance" of Pneumococcus (2) was discussed and methods were given for the isolation of the specific substances of Types II and III pneumococcus. In the present communication refinements of these methods are described. With their aid it has been possible to obtain both of these substances free from nitrogen and possessing much greater activity when tested with the homologous immune sera than did the products previously isolated. Further data are presented concerning the nature of the polysaccharides with which these substances appear to be identified.

The isolation of a specific substance from Type I pneumococcus is also described, and it is shown that this substance, while apparently also a sugar derivative, contains nitrogen and differs also in other respects from the specific substances isolated from the other two fixed types of pneumococci.

EXPERIMENTAL.

1. The Soluble Specific Substance of Type I Pneumococcus.

The original method of purification of the soluble specific substance was modified after numerous trials to fit the properties of the Type I substance.

8 day cultures of Type I pneumococcus in meat infusion phosphate broth are concentrated in 21 liter lots on the water bath to a volume of 1.2 to 1.5 liters. The concentrate is precipitated with 1.4 volumes of alcohol and put through the

initial 3 layer separation as described in Paper I. The middle layer[1] is dissolved as well as possible in 200 to 300 cc. of water and the mixture is centrifuged and the precipitate washed twice with small amounts of water. The clear solution and washings are kept on ice and combined with subsequent lots until 300 to 325 liters have been worked up. After solution of the deposit of salts the total concentrate, at a volume of about 4.5 to 5.0 liters, is treated with 1:1 hydrochloric acid until strongly acid to Congo red. The resulting precipitate is allowed to settle for several hours in the cold, after which as much of the supernatant liquid as possible is siphoned off and the remainder cleared by centrifugation. The precipitate is washed once with 0.01 normal hydrochloric acid and again separated by repeated centrifugation at high speed. The clear solution and washings, at a volume of 5 to 6 liters, are now precipitated with 7 to 8 liters of chilled alcohol and allowed to stand overnight in the cold. A test portion of the supernatant liquid, when neutralized and boiled down to small volume, should give no immediate precipitate with Type I antiserum. As much as possible is siphoned off and the crude specific substance separated from the remainder by centrifugation. The precipitate is then washed in the centrifuge bottles with 0.5 normal acetic acid, in which the isoelectric Type I substance is insoluble, while most of the accompanying glycogen or erythrodextrin dissolves. After several hours in the cold the mixture is centrifuged and the precipitate taken up in water. The specific substance is dissolved by addition of sodium hydroxide until the mixture is faintly alkaline to litmus, the volume is adjusted to about 700 cc., and the mixture is centrifuged at high speed until clear. The precipitate is washed with 50 cc. of water and again centrifuged. To the solution and washings are added 20 gm. of sodium acetate, and when this is dissolved 400 cc. of alcohol are added, with vigorous stirring, and the mixture is allowed to stand overnight in the cold. In relatively concentrated solution and in the presence of sufficient salts the Type I specific substance is quantitatively precipitated by 0.5 volume of alcohol in the cold. The use of sodium acetate is advantageous as it is not thrown out by alcohol at the concentrations used. The precipitate is centrifuged off, water is added up to a volume of about 500 cc., the mixture is made slightly alkaline again if necessary, and is finally centrifuged to remove any insoluble material. The solution is then stirred mechanically and the specific substance is rendered insoluble by the careful addition of 16 cc. of glacial acetic acid. After several hours in the cold it is centrifuged off and redissolved with the aid of sodium hydroxide as before, except that the volume should now be about 400 cc. 15 gm. of sodium acetate are added, and the specific substance is precipitated in the cold with 200 cc. of alcohol. After having stood overnight it is centrifuged off and redissolved in enough water to bring the volume to about 250 cc., using enough sodium hydroxide to make

[1] Centrifuged precipitates containing the soluble specific substances are usually compact and gummy, and care must be taken to smooth out all lumps when the deposit is redissolved.

the reaction slightly alkaline. The solution is chilled, stirred mechanically, and treated with an equal volume of barium hydroxide solution in equilibrium with the crystalline phase at about 50°. Most of the specific substance separates at once, and the mixture may be centrifuged after several hours in the ice box. Under optimal conditions the precipitation is complete, but occasionally up to about 15 per cent of the specific substance may remain in solution. If this is to be recovered it is best worked up separately, as coloring matter and other impurities are eliminated with it. The barium hydroxide precipitate is taken up in about 400 cc. of water and treated with 5 normal sulfuric acid until, after all lumps are smoothed out, the reaction remains acid to Congo red. The mixture is then centrifuged, and the precipitate of barium sulfate washed twice with very dilute sulfuric acid. The supernatant solutions are neutralized, and if opalescent, may be passed first through a Berkefeld V candle and then through the W grade. The solution is concentrated *in vacuo* to about 200 cc., made faintly alkaline with sodium hydroxide, and precipitated in the cold with 100 cc. of alcohol after the addition of 10 gm. of sodium acetate. The next day the precipitate is collected in the usual way, redissolved in water, and at a volume of about 200 cc. is again thrown out at the isoelectric point with 7 cc. of glacial acetic acid, using the same precautions as before. The precipitate is taken up in a little water and redissolved by addition of 1:1 hydrochloric acid. The volume is made up to about 125 cc. with water and hydrochloric acid, and the specific substance is then precipitated as the hydrochloride by the addition, with mechanical stirring, of 250 cc. of chilled redistilled alcohol. The centrifuged product is redissolved and reprecipitated in the same way and is then taken up in as small a volume as possible of cold water. The extremely viscous solution is then dialyzed in collodion bags in the ice box against successive changes of distilled water. The Type I specific substance is a weak base, and as the excess of hydrochloric acid diffuses out the hydrochloride is hydrolyzed and the isoelectric substance precipitates. Completion of the process and removal of the last traces of chlorine ion are accelerated by mixing the contents of the bags each time the water is changed. The mixture is finally centrifuged, the solution, which contains a little active material, is added to the next preparation, and the precipitate is washed with acetone, filtered on a hardened paper in a Büchner funnel, and dried. The yield should be from 2 to 3 gm.

The Type I specific substance obtained by this method contains 5 per cent of nitrogen and is often ash-free and colorless, although some preparations are still grayish in color and contain a little ash. In its isoelectric form the product is very sparingly soluble, but may be dissolved with the aid either of alkali or mineral acid. Concentrated solutions are extremely viscous. The specific optical rotation, +300°, is about the same on either side of the isoelectric point, which is

in the vicinity of pH 4. When boiled with a sufficient excess of mineral acid the substance is slowly hydrolyzed with formation of reducing sugars, which it has not yet been possible to identify fully. Reducing sugars also appear when the substance is treated with nitrous acid in the cold. One-half of the nitrogen is evolved at the same time, with simultaneous disappearance of the specific reaction. Since the specific substances of Types II and III pneumococcus are unaffected by nitrous acid under these conditions, it would appear that this half of the nitrogen, at least, is an integral part of the specific substance and is either linked to the reducing group of a sugar derivative, as Karrer believes is the case with the polyglucosamines (3), or else is so placed in the complex molecule that its removal causes some other type of scission. About one-half of the remaining nitrogen in the substance is given off within 15 minutes when the reaction mixture, freed from nitrous acid, is made alkaline and distilled with steam. The substance contains no sulfur or phosphorus.

When a concentrated aqueous solution of the hydrochloride of the specific substance is carefully treated with 1:1 hydrochloric acid a point is reached, at an acidity of 1.3 normal, at which the substance again precipitates, redissolving again if still more acid is added. Whether this indicates lactam formation, or some other reversible intramolecular change, is not yet clear.

$\frac{1}{2}$ per cent solutions, prepared by dissolving the specific substance in dilute acid and bringing it back past the isoelectric point with dilute sodium hydroxide, give precipitates with solutions of the following reagents: barium hydroxide, copper sulfate, silver nitrate, neutral and basic lead acetates, uranyl nitrate, and phosphotungstic acid. There is no color developed with iodine-potassium iodide solution, nor does the unhydrolyzed substance show reduction with Fehling's solution. The biuret, tannic acid, xanthroproteic, and ninhydrin tests are negative. With Millon's reagent a jelly is formed in the cold, but the precipitate redissolves on heating and no color is developed. Potassium permanganate is not immediately reduced.

After hydrolysis with nitric acid and subsequent oxidation mucic acid was obtained.

1.5 gm. of air-dry Preparation 39 were dissolved in 200 cc. of hot 1.4 normal nitric acid and boiled under a reflux condenser for 4 hours. The resulting green-

ish yellow solution was concentrated *in vacuo* to about 10 cc., and 4 cc. of concentrated nitric acid were added. The next day the mixture was boiled for a few moments and then stirred on a large clock-glass over boiling water until dry. The process was repeated with a few cc. of 1:1 nitric acid, after which the dry, white residue was taken up in acetone and allowed to stand overnight. The crystalline residue was filtered off, washed with a little acetone, and taken up in 5 cc. of normal ammonium hydroxide. The insoluble portion, apparently calcium oxalate, was discarded. Addition of 0.3 cc. of concentrated nitric acid to the filtrate resulted in the deposition of 0.21 gm. of mucic acid melting and decomposing at 216° with preliminary softening and blackening. The acetone mother liquors above were concentrated to dryness and taken up in water, when a second fraction of mucic acid was left behind. This was purified in the same way, yielding 0.07 gm., melting and decomposing at 215°. For analysis the two fractions were combined and recrystallized from water.

0.1008 gm., anhydrous, ash-free substance gave 0.1253 gm. CO_2 and 0.0442 gm. H_2O.

Calculated for $C_6H_{10}O_8$: C, 34.27 per cent; H, 4.80 per cent. Found: C, 33.9 per cent; H, 4.9 per cent.

From the aqueous mother liquors from which the second crop of mucic acid was obtained was isolated an acid potassium salt in a yield of 0.191 gm. and containing 22.3 per cent of potassium.

A repetition of the oxidation in the cold with 1 gm. of Preparation 41 gave after 1 week a deposit of mucic acid weighing 0.115 gm. after two recrystallizations.

Since the Type I specific substance gives a positive test for glucuronic acid with naphthoresorcinol it is possible that galacturonic acid is present as a part of the molecule, for this would yield mucic acid on oxidation. That the mucic acid does not arise from oxidation of galactose itself is indicated by the difficulty with which an osazone is formed from the products of hydrolysis, although crystallization of the osazone formed might also be hindered by the complexity of the reaction mixture.

While the Type I specific substance as at present obtained probably does not represent a single, chemically pure compound, it would nevertheless seem as if the major portion of the impurities had been removed. As will be seen from Table I, in which the figures given are calculated to the ash-free basis, the total nitrogen, the nitrogen and reducing sugars liberated on treatment with nitrous acid, and the optical rotation are remarkably constant in the majority of instances, although the preparations listed were isolated in different ways.

29B was prepared from Type I pneumococci themselves, instead of the autolyzed culture fluid, by solution in diluted bile, removal of the "nucleoprotein" with acetic acid, and adsorption of the specific substance on alumina, as described in the case of Type II pneumococcus (1).

In 36 and 37 the use of barium hydroxide was omitted, necessitating a more laborious fractionation. 37A was prepared from 37 by adsorption on a particularly active form of alumina (Alumina A) prepared according to the directions of Willstätter and Kraut (4).

41B_2 was obtained from 41 by interaction with neutralized formalin in slightly alkaline solution. Discharge of the phenolphthalein color indicated that the specific substance combined with the aldehyde. However, fractional precipitation of the resulting substance with alcohol (B_2 being the second fraction) failed to yield a product with different properties. That the combination with formaldehyde was a labile one was indicated by the fact that the product recovered, when once more treated as before with formalin, again showed an increase in the acid reaction, indicating that any formaldehyde combined with the specific substance had been eliminated in the process of isolation (addition of cold dilute hydrochloric acid and precipitation with alcohol).

43A was obtained by digestion of the product of the third alcoholic precipitation (see method) with an amount of ice-cold water sufficient to dissolve only a portion of the specific substance. The mixture had a total volume of 300 cc. in this case, and the specific substance was reprecipitated with alcohol and then purified with the aid of barium hydroxide. The residue from the A fraction was worked up in the usual way as 43B, while the part of it not precipitated by barium hydroxide, but separating when the alkaline mother liquors were acidified with acetic acid, is represented by 43C. It will be seen from Table I that each of these fractions gave the same analytical values, and the physical properties corresponded equally closely.

Finally, 44A, after precipitation with barium hydroxide, removal of the barium, and precipitation with alcohol, was purified twice over the sparingly soluble hydrochloride mentioned above. It was hoped that precipitation in this way by hydrochloric acid without the assistance of alcohol would result in the removal of nitrogenous or carbo-

TABLE I.

Preparation No.	Specific rotation.	Total N.	Amino N.	Reducing sugars on acid hydrolysis.*	Reducing sugars by HNO_2.	C	H	Ash.	Precipitation with anti-pneumococcus serum.†
Type I pneumococcus.									
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
29B	+301°	4.8	2.7	31.8		43.3	5.8		1:4,000,000
36	+304.5°	4.6‡	2.4	31.6				1.5	1:6,000,000
37	+295.5°	5.1	2.6	27.2				0.7	1:6,000,000
37A	+287°	4.1‡	2.5		31.7			7.9	1:6,000,000
38A	+310°	5.0	2.6	28.6	28.5			1.8	1:6,000,000
39	+304°	4.4‡	2.5		27.1			1.6	1:6,000,000
41	+303°	5.0	2.6		26.0			0.0	1:8,000,000
$41B_2$	+279°	4.4‡	2.5		23.9				
43A	+303°	5.0	2.5		28.8			1.2	1:6,000,000
43B	+300°	4.9	2.5	28.4	28.5			0.0	1:6,000,000
43C	+300°	5.0	2.6		28.0			0.0	1:6,000,000
44A	+300°	5.0	2.6		28.5	43.3	5.5	0.5	1:8,000,000
Type II pneumococcus.									
					Acid equivalent.				
25	+63.2°	0.18		80.3				1.9	1:3,000,000
25A	+70.2°	0.0		68.4	1302	45.8	6.4	0.35	1:6,000,000
25B	+56.7°	0.0			946				1:2,000,000
25C	+72.2°	0.12		67.6	1190				1:5,000,000
26A	+75.2°	0.0		69.1	1258			1.6	1:6,000,000
$26A_1$	+72.8°	0.0		68.2	1252			0.0	1:5,000,000
26B	+54.4°			56.0	1105				
Type III pneumococcus.									
30	−37.3°			73.0	330				1:6,000,000
30I	−30.9°	0.0		73.3	347				1:5,000,000
30A	−35.1°	0.0		71.0	339			0.0	1:6,000,000
33	−30.5°	0.0		73.0	343			0.0	
33I	−32.5°	0.0		74.5	341			0.0	
33II	−34.0°	0.0		75.5	340	42.7	5.3		
33B III	−30.8°	0.0		72.5	358			0.0	1:6,000,000

* Calculated as glucose.

† 2 hours at 37° and overnight in the cold.

‡ Micro Kjeldahl determinations using aeration method for collecting NH_3. Results about 10 per cent low. In the other cases Pregl's method was used.

hydrate material that would otherwise be precipitated by the alcohol customarily added. However, this preparation checked closely with the others in optical rotation, carbon, hydrogen, and nitrogen, and in the other quantitative and qualitative tests used.

2. Type II Pneumococcus.

A. Further Purification of the Type II Soluble Specific Substance.

The specific substance from 312 liters of broth culture was isolated according to the method given in Paper II. After the third precipitation with ammonium sulfate the deposited material was redissolved in hot water, run through a Berkefeld W candle to remove opalescence, and concentrated to 150 cc. The solution was cooled to 0°, acidified with 35 cc. of 1:1 hydrochloric acid, stirred mechanically, and precipitated by the addition of 350 cc. of chilled redistilled alcohol. After 2 hours in the ice box the precipitate was centrifuged off in the cold and redissolved in 75 cc. of cold water. The precipitation process was then repeated with 18 cc. of 1:1 hydrochloric acid and 300 cc. of alcohol. The precipitate, after centrifuging, was redissolved in 100 cc. of water, rinsed into a collodion bag with 5 cc. of normal hydrochloric acid, and dialyzed in the cold against distilled water until free from chlorine ion. The solution was concentrated to 50 cc. *in vacuo* and stirred into 600 cc. of redistilled acetone. After having stood overnight the precipitate was collected on hardened paper, washed with redistilled acetone, and dried. The yield was 3.3 gm. in the case of Preparation 26A, and 2.4 gm. for 25A.

The supernatant from the initial acid-alcohol precipitation still reacted strongly with Type II antiserum and was accordingly precipitated with 0.5 volume of ether. The material deposited was dissolved in 20 cc. of water and, after addition of a little hydrochloric acid, was dialyzed as in the previous instance, concentrated to 20 cc., and poured into 12 volumes of acetone. 0.6 gm. was recovered (26B) in one case and 0.5 in another (25B).

In an attempt to push the purification further a portion of 26A was again put through the acid-alcohol precipitation process. The product, $26A_1$, gave, however, the same analytical values as that from which it was derived.

From a comparison of Preparation 25, Table I, in which these additional steps in the purification process were omitted, with 25A, 26A, and $26A_1$, it will be seen that the remaining nitrogen in the specific substance has been eliminated, all but traces of ash have been removed, a fraction (25B and 26B) has been separated containing impurities of lower optical rotation, and the highest dilution at which the substance reacts with its homologous serum has been increased. In order to reduce the analytical error to a minimum micro Kjeldahl determinations were run on 25 mg. of substance in the case of 26A.

B. Confirmation of Glucose as the Chief Sugar Constituent of the Type II Specific Substance.

In the previous papers it was shown that glucosazone could be obtained from the hydrolysis products of the Type II soluble specific substance. Although the hydrolyzed material showed a specific rotation of about +55°, indicating that the resulting hexose was probably glucose, the possibility that the sugar might have been mannose or fructose was by no means excluded, owing to the possible presence of other optically active substances.

In order definitely to determine this point, a portion of the specific substance was hydrolyzed and subsequently oxidized.

0.5 gm. of Preparation 25A was dissolved in 25 cc. of water and boiled for 5 hours under a reflux condenser with an equal volume of normal nitric acid. The solution was evaporated *in vacuo* to 3 to 4 cc., treated with 2 cc. of concentrated nitric acid, and allowed to stand at room temperature overnight. The mixture was then boiled for 2.5 minutes over a flame, and poured on to a large watch-glass, stirred, and quickly evaporated over a boiling water bath. A thick paste was obtained and this was twice evaporated with water to expel the last traces of nitric acid, dissolved in 2 cc. of water, made strongly alkaline with 40 per cent potassium hydroxide solution, and reacidified with glacial acetic acid. The mixture was cooled in ice water, and after 24 hours in the cold the crystals of potassium acid saccharate which had separated were filtered off. 0.1 gm. was recovered. This was recrystallized from 1.5 cc. of water, yielding 0.062 gm. of the purified salt.

0.0602 gm. substance gave 0.0210 gm. K_2SO_4.

Calculated for $KOOC(CHOH)_4COOH$: K, 15.75 per cent. Found: 15.65 per cent.

The isolation of potassium acid saccharate through the oxidation of the hydrolytic products of the Type II soluble specific substance, together with the isolation of glucosazone previously reported, leaves little doubt that the units from which the polysaccharide is built up are those of glucose.

C. Preparation of a Triacetate of the Type II Soluble Specific Substance.

If the true soluble specific substance were actually a polysaccharide it should be possible to convert it into an acetyl derivative just as the celluloses and starches may be acetylated. On the other hand,

if the polysaccharide present were an impurity the altered solubilities of an acetyl derivative might be expected to facilitate its removal from the actual specific substance.

0.5 gm. of Preparation 25A was accordingly dried to constant weight *in vacuo* and shaken overnight at room temperature with a mixture of 2.5 cc. of dry acetic anhydride and 6 cc. of dry pyridine. Since no reaction appeared to have taken place the tightly stoppered bottle containing the mixture was heated in a water oven for 24 hours. The resulting suspension, which had darkened somewhat, was poured into ice water but most of the solid failed to dissolve. It was therefore filtered off, macerated with water until the washings failed to give a specific test with Type II antiserum, and shaken with 200 cc. of water and a few glass beads until it had disintegrated into a very fine powder. After centrifugation the supernatant fluid gave only a weak test with immune serum. The residue was next washed with 50 cc. of methyl alcohol, which removed most of the coloring matter, and was dried *in vacuo*. 0.7 gm. of an amorphous, fluffy, tan powder was recovered.

0.1009 gm. substance gave 0.1864 gm. CO_2 and 0.0503 gm. H_2O.

Calculated for $[C_6H_7O_5(OCOCH_3)_3]_x$: C, 49.95 per cent; H, 5.60 per cent. Found: C, 50.4 per cent; H, 5.6 per cent.

0.1084, 0.1190 gm. substance hydrolyzed with 0.5 N NaOH, neutralized 9.70, 10.90 cc. 0.1 N NaOH respectively. Calculated: 11.30, 12.40.

The triacetate was found to be practically insoluble in water and the common organic solvents. It dissolved, however, when warmed with dilute sodium hydroxide, the neutralized solution giving a heavy precipitate with antiserum. On account of the extreme insolubility of the triacetate it was difficult to determine definitely whether or not it was specific in itself. However, the large yield and the fact that it again yields a specifically reacting substance on hydrolysis showed that no separation of the polysaccharide from the actual specific substance had been effected.

D. Recovery of the Type II Specific Substance from Its Precipitate with Homologous Antibody.

In Paper II it was shown that the Type II specific substance could be recovered from its precipitate with immune serum. Owing to the difficulty met with in separating the polysaccharide from large amounts of serum protein it seemed advisable to repeat this experiment, using instead of the serum an antibody solution purified essentially according to Felton (5).

1.5 liters of Type II antipneumococcus serum were gradually added with stirring to 28.5 liters of well chilled N/100 acetic acid-sodium acetate buffer at pH 5. After having stood overnight the precipitate was centrifuged off, dissolved in 750 cc. of 0.85 per cent salt solution, centrifuged once more, and again added to 19 volumes of the same buffer solution. The resulting precipitate, which contained a large proportion of the antibodies in the original serum, was finally collected as before and dissolved in 1.5 liters of saline.

To this solution was slowly added 0.2 gm. of Preparation 25A dissolved in 500 cc. of saline. After 2 hours in the incubator at 37° and standing in the ice box overnight the precipitate was centrifuged off and was washed with 150 cc. of saline, centrifuged again, and suspended in 150 cc. of a citrate-phosphate-borate buffer solution prepared according to Northrop (6). The mixture was digested at 37° with 1 gm. of trypsin added in small portions at intervals until a clear solution was obtained. This was then treated with 2 volumes of alcohol and the resulting precipitate was suspended in water and treated with dilute sodium hydroxide solution until it just dissolved. The solution was heated to boiling and neutralized with dilute acetic acid, causing the separation of a coagulum. The supernatant liquid containing the specific substance was evaporated to complete dryness and taken up in boiling water. A small amount of insoluble protein material was centrifuged off and the supernatant liquid was dialyzed until free from salts and repeatedly evaporated and extracted with water. With each evaporation a small amount of insoluble protein material was eliminated. The extract was finally saturated with ammonium sulfate, whereby a heavy yellow protein precipitate separated. This was centrifuged off, redissolved in a little water, reprecipitated with ammonium sulfate, and again centrifuged. The supernatant liquid containing the soluble substance was again dialyzed until free from sulfate. The solution was then concentrated to 5 cc. and precipitated with 10 cc. of alcohol and a few crystals of sodium acetate. The inactive supernatant liquid was discarded and the precipitate was twice reprecipitated by alcohol from a volume of 5 cc. The substance was finally precipitated from the same volume at 0° by the addition of 1.5 cc. of 1:1 hydrochloric acid and 2 volumes of alcohol. It was then dissolved in a few cc. of water and added to a large excess of acetone. The yield was 0.12 gm.

The recovered specific substance (25C) failed to give the biuret reaction and contained only 0.12 per cent of nitrogen, whereas in the original experiment with serum the nitrogen content was 1.0 per cent. In its other properties it agreed well with the highly purified material used to precipitate the antibody solution.

3. The Soluble Specific Substance of Type III Pneumococcus.
A. Further Attempts at Purification.

A preparation of the Type III specific substance which had been twice purified by precipitation with hydrochloric acid (see Paper II) was reprecipitated three times in this manner in order to determine whether any additional impurities could be eliminated. However, the preparations, 33, 33I, and 33II checked well in analytical values with each other and with previous preparations, except that no nitrogen was found, although in the case of 33I 35.5 mg. of substance were used for each micro Kjeldahl determination.

Since it had been found that the Type III substance gave a precipitate when barium hydroxide in excess was added, an additional purification of the substance was attempted over the barium salt.

0.5 gm. of Preparation 33 was finely pulverized and suspended in 25 cc. of water. The mixture was heated to boiling under a reflux condenser and to it was added enough 0.2 normal barium hydroxide solution to keep the reaction just alkaline to phenolphthalein. The solution was centrifuged from a few particles which had failed to dissolve, and the supernatant liquid, which contained practically all of the substance as a soluble barium salt, was treated with a slight excess of saturated barium hydroxide solution. After the resulting heavy flocculent precipitate was centrifuged off the supernatant liquid gave only a slight precipitate with immune serum. The barium salt was washed once with water containing a small amount of barium hydroxide and was then dissolved in about 350 cc. of hot water and treated cautiously with sulfuric acid until the supernatant liquid showed neither barium nor sulfate ions. The barium sulfate was centrifuged off and the supernatant liquid concentrated *in vacuo* to small bulk and dialyzed in a collodion bag. After further concentration *in vacuo* the solution was added to 10 volumes of redistilled acetone. The precipitate was sucked dry on a filter, washed with acetone, and dried *in vacuo* over phosphorus pentoxide until constant in weight, a process which rendered the recovered Type III acid insoluble. The yield was 0.43 gm.

A comparison of the properties of Product 33B III with those of 33 (Table I) indicates that precipitation of the Type III acid as the barium salt does not effect a fractionation.

An attempt was also made as in the case of Type I to remove possible impurities by adsorption of the specific substance on the highly reactive Type A alumina prepared according to the method of Willstätter and Kraut (4).

As a result of preliminary experiments it was found that at a pH below 6.0 1 gm. of gel (calculated as Al_2O_3) adsorbed approximately 0.35 gm. of polysaccharide. At a pH higher than 7.0 the adsorption of the Type III substance did not reach completion.

0.5 gm. of Preparation 30 was dissolved in the equivalent amount of N/14 sodium hydroxide. To the solution was added a suspension of 1.5 gm. of alumina and the volume was made up to 750 cc. The pH was adjusted with N/5 hydrochloric acid to 5.0 and the suspension was shaken for 1 hour, after which the supernatant fluid gave a negative precipitin test. The mixture was centrifuged and the precipitate washed once with water. It was then extracted twice with N/5 sodium carbonate solution and the extract, after neutralization, was concentrated *in vacuo*, dialyzed in parchment, again concentrated *in vacuo*, and dialyzed in collodion until free from chloride ion. The solution was then further concentrated *in vacuo* and precipitated from a volume of 15 cc. by 5 cc. of 1:1 hydrochloric acid and 40 cc. of redistilled alcohol at 0°. The specific acid was washed free from chlorides with 50 per cent alcohol and finally with acetone. 0.36 gm. of dry material was recovered.

A comparison of Product 30A with the starting material shows that adsorption of the specific substance on alumina did not result in the separation of any significant amount of impurity, a result also given by the other methods of purification attempted.

The variations shown by the optical rotation of the Type III substances listed in Table I may perhaps be explained by the fact that in each instance the insoluble acid was dissolved in a slight excess of dilute alkali. It was found that alkaline solutions of the substance suffered a slow decrease in optical rotation, followed by a slow return to the original value after acidification. For instance, in one case a portion of Preparation 33II, with an initial $[\alpha]_D$ of $-34.1°$ showed a drop in 0.7 per cent sodium hydroxide solution to a value of $-21.2°$, this returning to $-36.0°$ after acidification.

B. Hydrolytic Products of the Type III Specific Acid.

As a preliminary step in the study of the products of hydrolysis, the relation between the specificity, the time of hydrolysis, the percentage of reducing sugars, and the optical rotation was studied.

0.0836 gm. of dried Preparation 30I, of which the $[\alpha]_D$ was $-30.9°$, was dissolved in 0.55 cc. of concentrated sulfuric acid at $-10°$ and allowed to stand at this temperature for 1 hour. The viscous solution was then diluted to 21 cc.

to give an approximately normal solution of acid, and, before hydrolysis on boiling, its optical rotation, reducing power, and specificity were tested. The values observed are to be found in the following table.

Period of boiling.	$[\alpha]_D$	Reducing sugars (calculated as glucose).	Reaction with Type III antipneumococcus serum.
min.		*per cent*	
0	−15.9°	0.0	++
140	+8.8°	54.8	±
180	+15.4°	61.0	−
300	+22.6°	69.4	−
360	+23.4°	65.5	−

It is thus evident that in boiling normal sulfuric acid the specific reaction of the Type III acid persists up to the 3rd hour, and that the maximum reducing power is attained in about 5 hours.

6 gm. of Preparation 30I with a water content of 7.6 per cent, were finely pulverized and slowly added to a mixture of 30 gm. of concentrated sulfuric acid and 10 cc. of water at 0°. The acid was stirred slowly with a turbine and after a short time 10 cc. more of the sulfuric acid mixture were added. After 2 hours only a few lumps remained and the mixture was allowed to stand in the ice box overnight. It was then poured into 800 cc. of water and boiled under a reflux condenser for 5 hours, at which time the reducing power showed a constant value. The sulfuric acid was removed quantitatively with barium hydroxide. The barium sulfate was centrifuged off, and the liquid concentrated *in vacuo* to 100 cc., boiled with calcium-free Norite, and filtered. The colorless filtrate was treated with an excess of basic lead acetate and centrifuged. The precipitate was washed once with water containing a little basic lead acetate, three times with alcohol, and was then filtered off, washed with acetone, and dried. 10.7 gm. of the lead salt were recovered in this way.

The supernatant from the basic lead acetate precipitate contained a strongly reducing substance (0.96 gm. calculated as glucose) and was accordingly treated with hydrogen sulfide to remove lead, filtered, and concentrated, finally, *in vacuo* to dryness in a desiccator. After a few days crystals could be seen with a hand lens but on account of the difficulty in isolating them in appreciable quantities the whole was treated with 6 cc. of 1:1 nitric acid and oxidized as described previously in this paper.

0.15 gm. of recrystallized potassium acid saccharate was readily obtained.

0.0804 gm. substance gave 0.0282 gm. K_2SO_4.

Calculated for $COOK(CHOH)_4COOH$: K, 15.75 per cent. Found: K, 15.73 per cent.

A similar sugar fraction from the hydrolysis of 4.0 gm. of Type III acid, containing 0.6 gm. of sugar calculated as glucose was de-leaded with hydrogen sul-

fide and treated in a volume of 50 cc. with 1.3 gm. of phenylhydrazine dissolved in 3 cc. of 50 per cent acetic acid. After warming for 1 hour on the water bath the copious deposit of yellow crystals was filtered off, washed with 10 cc. of methyl alcohol to remove tar, filtered, and dried. 0.40 gm. of osazone was obtained, melting and decomposing at 203–204°. A solution of 0.0760 gm. in 7.6 cc. of pyridine-alcohol mixture showed, in a 0.5 dm. tube, a rotation of −0.33°. After 2 days the reading had decreased to −0.12°, mutarotation in this direction being a characteristic of glucosazone (7). Recrystallized for analysis, the osazone decomposed at 206–210°.

Calculated for glucosazone, $C_{18}H_{22}O_4N_4$: N, 15.45 per cent. Found: 15.42 per cent.

The salt formed as described above by the addition of basic lead acetate to the hydrolysis product was suspended in about 300 cc. of water and treated with hydrogen sulfide. The lead sulfide was removed and the filtrate concentrated *in vacuo* to small volume, boiled with Norite, filtered, and the filtrate evaporated to complete dryness over phosphorus pentoxide. 3.1 gm. of a white glassy mass remained. It was readily soluble in water, difficultly soluble in alcohol, and gave a strong glucuronic acid test with naphthoresorcinol. However, its non-crystalline nature, its insolubility in alcohol, and the difficulty of isolating from it a bromophenylosazone did not support its being glucuronic acid itself. Moreover its $[\alpha]_D$ was +12.3° (0.3372 gm. in 20 cc. of water), whereas the specific rotation of glucuronic acid is +19.1°. It also had a reducing power of only 48.0 per cent, calculated as glucose. This value could be increased about 10 per cent by boiling with 0.5 normal hydrochloric acid, while boiling with hydrobromic acid of the same strength increased the reducing power about 15 per cent and concentrated hydrochloric acid in the cold caused an increase of only 7 to 8 per cent. Saccharic acid could not be isolated from the product by oxidation with bromine. The reducing group was also readily oxidized by nitric acid, but the product again showed remarkable stability toward further hydrolysis.

It is thus quite certain that the second component of the hydrolysis products of the Type III acid is not glucuronic acid itself, although there is evidence that it consists of glucuronic acid combined with some other substance, perhaps a hexose derivative. It is hoped to clear up this point when more material becomes available.

As to the portion of the hydrolysis products not precipitated by basic lead acetate, however, there can be no doubt that the principal substance present is glucose, owing to the high yield of glucosazone obtained, and to the isolation of acid potassium saccharate on oxidation of the sugar.

C. Conversion of the Insoluble Type III Acid into a Soluble Form.

When the insoluble form of the Type III acid, obtained in the usual way, is boiled with water it gradually enters into solution.

0.1 gm. of Preparation 30I was shaken for 1 hour with 200 cc. of water at 50°. Since only a swelling of the granules of the specific substance occurred, the mixture was diluted to a volume of 500 cc. and boiled under a reflux condenser for 1½ hours. The resulting clear solution was filtered as a precaution and concentrated, finally, in a vacuum desiccator until the volume was 5 cc. The solution, which was still clear, was thrown into 100 cc. of acetone and filtered. The precipitate was washed with acetone and absolute alcohol and was dried at room temperature over sulfuric acid at atmospheric pressure until a constant weight of 0.09 gm. was reached. The substance thus recovered was a white amorphous powder soluble in water, but after it was again dried *in vacuo* it gradually passed into the insoluble derivative. $[\alpha]_D$ of the soluble form was $-31.9°$, and the acid equivalent (352) was in agreement with the values obtained by solution of the insoluble form in dilute alkali and subsequent neutralization.

Whether the reversible equilibrium between the soluble and insoluble forms is one involving simple hydration and dehydration, or one depending on the opening and closing of a lactone or anhydride grouping cannot be decided with the evidence at present available.

DISCUSSION.

The following data are given in order to summarize briefly the properties of the soluble specific substances of the three fixed types of Pneumococcus, in the state of purity thus far attained.

The soluble specific substance of Type II pneumococcus is apparently a weakly acidic, nitrogen-free polysaccharide made up chiefly of glucose units. Its acid equivalent is about 1250 and the specific optical rotation is about $+74°$. It is not precipitated by barium hydroxide or heavy metal salts with the exception of basic lead acetate and uranyl compounds. It reacts at a dilution of 1:5,000,000 with Type II antipneumococcus serum but does not precipitate Type I and Type III antisera at a concentration of 1:400. The substance is converted by acetic anhydride and pyridine into a very sparingly soluble triacetyl derivative.

The Type III soluble substance, while also isolated as a nitrogen-

free polysaccharide, is a strong acid with an acid equivalent of about 340, and is made up not only of glucose units but also those of either glucuronic acid or a derivative of this acid. It rotates the plane of polarized light about 33° to the left. It is precipitated by barium hydroxide in excess and by heavy metal salts, and is also rendered insoluble by the addition of strong hydrochloric acid. In as high a dilution as 1:6,000,000 it still reacts with Type III antipneumococcus serum.

The Type I soluble specific substance also appears to be a sugar derivative, but differs from the other two substances in its lower percentage of sugar liberated on hydrolysis and in containing nitrogen as an apparently essential component. It rotates the plane of polarized light about 300° to the right, is a strong acid and a weak base, and is very sparingly soluble at its isoelectric point, which lies at about pH 4. In spite of a nitrogen content of 5.0 per cent the substance gives none of the usual protein color tests. One-half of the nitrogen is liberated on treatment with nitrous acid and reducing sugars appear at the same time, while the specific reaction vanishes. Under the same conditions the Type II and Type III substances are unaffected by nitrous acid. The substance gives the color reaction for glucuronic acid with naphthoresorcinol, but yields mucic acid on oxidation, indicating a relationship to galactose. Since the carbon and hydrogen contents of the substance are close to the theoretical values for polysaccharides it appears possible that in it a nitrogenous sugar derivative is linked to galacturonic acid through the reducing group of the latter. Further evidence on this point will be sought. The Type I substance is precipitated by barium hydroxide in excess, by heavy metal salts, and by phosphotungstic acid. In the specific precipitin reaction with homologous Type I antipneumococcus serum it can be detected in dilutions as great as 1:6,000,000 while at a concentration of 1:400 it gives a faint cloud with Type III antiserum.

The three polysaccharides contain no sulfur or phosphorus and differ from the starch-glycogen group of carbohydrates in giving no color with iodine and in their resistance to the ordinary carbohydrate-splitting enzymes.

It would be, of course, idle to assume that in their present state of purity, each of the specific substances represents a definite chemical

compound. However, in the case of the three fixed types of Pneumococcus three totally distinct substances have been isolated from cultures grown in the same medium. Successive preparations of the specific substance have in each instance been quite uniform regardless of the widely different methods employed in the process of purification. Moreover, substances reactive to the same degree with homologous antisera have been derived both from the microorganisms themselves and from autolyzed broth cultures.

It is thought that these and other considerations based on the data presented warrant the belief that the three polysaccharides isolated represent the actual specific substances, stripped of at least a large portion of accompanying impurities, and that they do not merely represent inert material carrying an extremely minute amount of the true specific compounds.

If this be accepted it may be concluded that the soluble specific substance of each of the three fixed types of Pneumococcus is a distinct chemical substance, differing in many striking particulars from the corresponding product elaborated by the other two types, but having in common the properties of polysaccharide structure and of resistance to enzyme action. Each substance breaks down on hydrolysis into reducing sugars, a part of which, at least, is peculiar to itself. The Type I substance differs sharply from the other two in containing nitrogen and in possessing basic as well as acid properties, while of the other two substances, the Type II is a dextrorotatory weak acid and the Type III a levorotatory strong acid. Especially striking is the occurrence of specific substances of such widely differing properties in microorganisms as closely related as the three fixed types of Pneumococcus.

The immunological significance of the specific substances has been discussed by the writers in a recent paper (8) and will therefore not be touched upon in the present communication.

Many of the questions raised in the course of the work are still under investigation and the specific substances of other microorganisms are being studied.

SUMMARY.

1. Refinements in the methods for the isolation of the soluble specific substances of Types II and III pneumococcus are described.

These improvements have resulted in the isolation of the end-products in a form free from nitrogen and of enhanced activity with immune serum.

2. The soluble specific substance of Type I pneumococcus is described and shown to differ sharply from the corresponding substances of the other two types, each of which, in turn, differs from the other.

3. Progress is reported on the identification of the sugar units from which the polysaccharides are built up.

4. The evidence so far accumulated is believed to favor strongly the view that the polysaccharides isolated are the actual specific substances of Pneumococcus.

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